INTERACTION OF ADENO-ASSOCIATED VIRUS (AAV) WITH THE TRANSFORMING GENES OF ADENOVIRUS

BY

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This dissertation is dedicated to my mother Naomi, and my father Al. Without their never ending love and support this work would never have begun.

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ABBREVIATIONS USED

AAV . . . . . Adeno-associated Virus

ABM . . . . . Aminobenzoxymethyl

Ad . . . . . Adenovirus

AGMK . . . . . African Green Monkey Kidney

ASV . . . . . Adeno-satellite Virus

BSA . . . . . Bovine serum albumin

CELO . . . . . Chicken Embryo Lethal Orphan

cpm . . . . . Counts per minutes

d . . . . . dalton

DBM . . . . . Diazobenzoxymethyl

DBP . . . . . DNA Binding Protein

DI . . . . . Defective Interfering

DMBA . . . . . Dimethylbenzanthrecene

DNA . . . . . Deoxyribonucleic Acid

dNTP . . . . . Deoxyribonucleoside triphosphate

EDTA . . . . . Ethelene diaminetetraacetic acid

ffu . . . . . Fluorescent focus unit

g . . . . . gravity

HEF . . . . . Hamster Embryo Fibroblasts

hr-mutants . . . . . Host-range Mutants

ICH . . . . . Infectious Canine Hepatitis Virus

IU . . . . . Infectious unit

K	1000 daltons
mA	Milliamps
MEM	Minimal Essential Medium
Moi	Multiplicity of infection
mRNA	Messenger RNA
N_D	Refractive Index
NHS	Normal Hamster Serum
PBS	Phosphate Buffer Saline
pfu	Plaque forming unit
^{32}Pi	Inorganic ^{32}Phosphate isotope
poly A	Polyadenylic Acid
RNA	Ribonucleic Acid
SDS	Sodium dodecyl sulfate
SV40	Simian Virus 40
T-Antigen	Tumor-antigen
TCID	Tissue Culture Infectious Dose
ts	Temperature Sensitive
UV	Ultraviolet
V	Volt
VP1	Viral Structural Protein 1
VP2	Viral Structural Protein 2
VP3	Viral Structural Protein 3

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INTERACTION OF ADENO-ASSOCIATED VIRUS (AAV) WITH THE TRANSFORMING GENES OF ADENOVIRUS

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The interactions between adeno-associated virus (AAV) and the transforming genes of adenovirus (Ad) have been characterized using Ad transformed hamster cells (H14b cell line) and host range mutants (hr) of Ad defective in expression of the Ad 58K tumor antigen.

AAV infection of H14b cells decreased their oncogenicity by approximately 3-fold. The tumors which arose were significantly smaller and had a longer latent period. AAV infection also altered some *in vitro* properties of the H14b cells and reduced cloning efficiency in methyl cellulose by 38-92% and the saturation density by 2-4 fold. The molecular basis of this inhibition appeared to be an inhibition of the level of the 58K tumor-antigen as determined by fluorescent antibody and immunoprecipitation studies. The reductions in the levels of tumor-antigens were not due to alterations or loss of the Ad genome within H14b cells but may reflect an alteration in transcription of Ad-specific mRNA.

Various early and late temperature sensitive mutants of Ad were able to help AAV DNA replication in either coinfections or in the rescue of AAV from latently infected human (Detroit 6) cells. Group I Ad host range mutants (early region 1a) were unable to replicate or rescue AAV while Group II Ad host range mutants (early region 1b) were able to help AAV DNA replication in coinfection but were unable to rescue AAV DNA in latently infected Detroit 6 cells. The failure of hr 6 to induce AAV DNA replication in latently infected Detroit 6 cells is of particular significance. This result demonstrates that in addition to Ad functions required for AAV DNA replication in a normal coinfection, one or more additional functions must be supplied by the Ad helper specifically to rescue AAV from latently infected cells. Since hr 6 cannot transform rat cells and does not synthesize the 58K tumor antigen, there may well be a role for the Ad 58K tumor antigen in the rescue of AAV.

CHAPTER I

INTRODUCTION

AAV Structure

The adeno-associated viruses (AAV), also known as adeno-satellite viruses (ASV), are members of the Parvoviridae family (for review see Berns and Hauswirth, 1979). These small DNA containing viruses are icosahedral with 12-20 capsomers (Mayor et al., 1965). The diameter of the capsid, as seen by electron microscopy, is 18-24 nm (Atchison et al., 1965; Smith et al., 1966; Crawford et al., 1969). AAV virions contain 80% protein, 20% DNA (Parks et al., 1967) and depending on the serotype, have a density in cesium chloride of 1.37-1.38 or 1.43 g/cm^3 (Smith et al., 1966; Parks et al., 1967; Crawford et al., 1969). Three major structural proteins have been found in the virion, VP1-VP3, in the ratio 80:10:10 respectively (Rose et al., 1971; Johnson et al., 1971). The single-stranded linear AAV DNA has a molecular weight of 1.4×10^6 d. Both the plus and minus DNA strands are packaged into separate virions at equal frequencies so purification of virion DNA usually results in the formation of duplex AAV DNA with 55% (G+C) due to the hybridization of complementary strands (Mayor et al., 1965; Rose et al., 1966; Parks et al., 1967; Mayor et al., 1969; Rose et al., 1969; Berns and Rose, 1970; Berns and Adler, 1972).

Biology of AAV

AAV is a defective virus which can replicate only in the presence of a helper adenovirus (Hoggan et al., 1966; Smith et al., 1966). When AAV infects a cell, it is uncoated in the nucleus where it can remain in an inactive state for a period of time and then be lost (Rose and Koczot, 1972; Berns, unpublished data) or produce a latent infection in which the AAV genome integrates into the host cell and remains for more than 100 passages (Berns et al., 1975; Handa et al., 1977; Cheung et al., 1980). Upon addition of helper adenovirus (Ad) to latently infected cells, infectious AAV virions are produced (Berns et al., 1975). All AAV's have a strict helper dependency; these include isolates of human, simian (for review see Hoggan, 1970), bovine, avian, and canine origin (Dutta and Pomroy, 1967; Domoto and Yanagawa, 1969; Luchsinger et al., 1970; Yates et al., 1972). It appears that AAV can replicate in any suitable cell if the helper Ad can replicate in that cell; for example, human AAV can replicate in canine kidney cells in the presence of infectious canine hepatitis virus (ICH) as the helper Ad (Hoggan et al., 1966) and human AAV has been shown to replicate in chick embryo fibroblasts when Chicken Embryo Lethal Orphan (CELO) virus is present (Blacklow et al., 1968).

There are four serotypes of AAV isolated from primates. AAV-2 and AAV-3 were isolated from Ad preparations grown in cells of human origin (Hoggan et al., 1966). AAV-1 was isolated from Ad stocks grown both in simian and human cells (Archetti and Bocciarelli, 1964, 1965), while AAV-4 has been isolated from African Green Monkey cells (Mayor et al., 1965; Parks et al., 1967).

The various primate serotypes share approximately 50% DNA sequence homology yet their native virion proteins are immunologically distinct (Rose et al., 1968; Hoggan, 1970; Koczot, M.S. Thesis, Univ. of Maryland, 1970). Antibodies raised against SDS disrupted virion proteins share immunological cross-reactivity demonstrating some relatedness between these proteins (Johnson et al., 1977).

AAV is a common virus in nature, with a high proportion of normal adults (70-75%) having antibodies to one or more serotypes, yet no disease states are known to be associated with the virus (Blacklow et al., 1967; Hoggan, 1970; Mayor et al., 1976).

AAV Replication

AAV is a defective virus which requires Ad coinfection in order to replicate. After simultaneous infection of permissive cells with AAV and adenovirus, adsorption occurs and during the first few hours AAV and adenovirus are transported into the nucleus (Rose and Koczot, 1972) where uncoating occurs (Berns and Adler, personal communication). AAV DNA synthesis is detectable 6-7 hrs post-infection followed 2 hrs later by AAV RNA synthesis. AAV proteins can be found by 14-15 hrs (Blacklow et al., 1967; Rose and Koczot, 1972; Carter, 1978). In cells preinfected for 10 hrs by adenovirus, AAV DNA and RNA synthesis occur 3-4 hrs post-infection (Blacklow et al., 1967; Carter, 1978).

Replication of AAV DNA seems to follow the model proposed by Cavalier-Smith (1974). AAV DNA has inverted terminal repetitions that contain palindromic sequences which can "snap back" to form hairpin structures that seem to be involved in the initiation of DNA synthesis (Gerry et al., 1973; Koczot et al., 1973; Berns and Kelly, 1974; Fife

et al., 1977; Spear et al., 1977; Hauswirth and Berns, 1977; Lusby et al., 1980). Replication of AAV DNA proceeds in the 5' to 3' direction with polymerization of DNA complementary to the parental strand (Straus et al., 1976; Berns and Hauswirth, 1978). Replication of the ends of AAV involves nicking of the parental strand within the inverted terminal repetition creating a new 3'-OH from which replication can proceed to the end of the molecule (Fife et al., 1977; Spear et al., 1977; Berns and Hauswirth, 1978; Lusby et al., 1980).

Various AAV transcripts have been mapped in cells coinfected with Ad and AAV. Four pairs of mRNA can be detected, each consisting of spliced and unspliced species. The spliced RNAs have the region mapping between 41-49 units (1 unit equals 1% of the genome) removed while all of the transcripts have polyadenylated termini mapping at 96 units. The predominant mRNA pair (87% of total AAV mRNA) maps with its 5' origin at 39 units and the other mRNA pairs map with origins at 5, 13, and 19 units respectively. The 5-96 transcript is 91% genome length and may be a precursor to the spliced RNA species. Another possibility is that there are separate promoters at 5, 13, 19, and 39 units for the various species (Carter et al., 1976; Jay et al., 1978; Laughlin et al., 1979; Green et al., 1980; Green and Roeder, 1980).

The AAV virion contains three major structural proteins: VP1 (62,000 d); VP2 (73,000 d), and VP3 (87,000 d), with a total molecular weight of 220,000 d. Assuming AAV is 4700 bases long, the theoretical coding capacity for AAV approaches 120,000 d of protein; this value is approximately half of what is actually found (Johnson et al., 1971; Rose et al., 1971). Two possibilities exist that can explain this paradox: 1) VP1-VP3 are coded for by the spliced mRNA species,

2) VP1-3 are derived from a precursor polypeptide. Evidence for multiple mRNA species comes from the work of Buller and Rose (1978) in which they demonstrated that in vitro translation of purified AAV mRNA resulted in the production of polypeptides comigrating with VP1, VP2 and VP3. These results can be interpreted as meaning that the various AAV proteins arise from translation of different mRNA species though these mRNA's may also share overlapping sequences or all three proteins are translated off one mRNA. Through this method the related proteins can share regions of amino acid sequences but arise from different transcripts.

Isolation of a 120,000 d precursor AAV protein from AAV infected cells has been reported (Johnson et al., 1977). This protein called VP0 could be chased into VP1, VP2, and VP3 over an 11 hour period. When VP1, VP2, and VP3 were compared antigenically through cross-immuno-precipitation reactions, they were shown to cross react sharing 72-91% antigenic similarities (Johnson et al., 1977). VP1, 2, and 3 share molecular similarities including isoelectric points ranging from 5.0-5.3. Two dimensional analysis of tryptic digests show there are at least 30 common peptides. VP1 has one unique peptide and shares one other with VP2 that is absent in VP3. VP2 and VP3 also share one peptide that is absent on the VP1 map, and VP3 has two unique peptides (Johnson et al., 1978; Lubeck et al., 1979).

Defectiveness of AAV

The efficient production of progeny AAV virions will only occur when a cell is infected with both AAV and helper Ad (Hoggan et al., 1966; Smith et al., 1966). Ad is the only virus that can serve as a complete helper for AAV. Herpesvirus has been shown to serve as a

partial helper for AAV DNA, RNA and protein synthesis though little infectious progeny AAV are made (Atchison, 1970; Blacklow et al., 1970; Blacklow et al., 1971; Boucher et al., 1971). Recently, Rose has obtained evidence for limited production of AAV progeny using herpesvirus as a helper (personal communication).

The defectiveness of AAV is not due to a very early event of virus-cell interaction because in the absence of helper virus, AAV will still adsorb normally, penetrate and enter the nucleus where uncoating occurs (Rose and Koczot, 1972; Berns, 1974). Purified AAV DNA will also enter the cell but will not replicate in the absence of helper Ad (Cheung, 1979). Both herpesvirus and Ad provide the functions necessary for AAV DNA, RNA and protein synthesis, but efficient assembly takes place only when adenovirus serves as helper.

Mutant Ad has been used to investigate the genes necessary for helper function. When U.V. treated Ad7 is used to infect cells, a 23 hr delay in Ad T-antigen expression is seen; when AAV is added to this system there is an identical delay in the expression of AAV antigens implying a close relationship between expression of Ad early functions and AAV replication (Blacklow et al., 1967). All temperature-sensitive (ts) mutants of Ad investigated have the ability to help AAV at both the restrictive and permissive temperatures. There is a possible exception to this in ts 94, an Ad31 maturation defective mutant which may not be able to help AAV assembly though AAV DNA and proteins are made normally (Ito and Suziki, 1970; Straus et al., 1976; Handa et al., 1976; Mayor and Young, 1978). Ad 5 ts125 and ts149 are both defective in their own DNA synthesis and in late RNA and protein synthesis but can synthesize T-antigen and transform cells at

the restrictive temperature (Ensinger and Ginsberg, 1972). These two ts mutants provide the necessary helper functions for AAV replication equally well at both the permissive and restrictive temperatures. These data imply that some but not all of the genes involved in Ad DNA replication are necessary to provide AAV helper function. These authors suggest an early function linked to Ad DNA replication provides help (Straus et al., 1976). Further evidence that Ad early functions provide help come from studies which show that microinjection of Ad2 early mRNA into Vero cells (monkey origin) promotes growth of AAV. In this system no Ad late functions are present (Richardson et al., 1980).

A system that seems to dissociate AAV replication from Ad DNA synthesis is the abortive growth of human Ad in African green monkey kidney cells (AGMK). When Ad infects AGMK cells, Ad T-antigen, DNA synthesis and RNA synthesis all occur but very little progeny Ad is produced. When AAV is added to this system AAV protein synthesis is blocked implying an Ad helper function is needed at yet another step in its replication (Friedman et al., 1970; Rose, personal communication).

A single report in the literature claims that AAV replication requires Ad early gene functions mapping in the left hand 7.2% in combination with human host cell factors (Handa et al., 1977). When a rat cell line (GY1) transformed with Ad12 Hind III-G fragment was fused with human KB cells, the heterokaryons created had the ability to replicate AAV, yielding infectious virus. The rat cell line GY1 or its normal parent cell 341 will not replicate AAV even in the presence of human Ad 12 virus indicating it was the combination of the human cell

factors supplied by the KB cells and the Ad early functions supplied by the Ad transformed rat cell that helped AAV replication (Handa et al., 1977; Handa et al., 1978). The significance of this is not clear because AAV is unable to replicate in Ad transformed cells expressing early functions such as T-antigen production (unpublished observation). This includes Ad transformed human, rat and hamster cell lines. Superinfection of these cells with Ad and AAV allows for productive infections (unpublished data).

Inhibitory Effects of AAV

Since the recognition that AAV is a defective virus and not a breakdown product of Ad, the presence of AAV in a mixed Ad-AAV infection has been shown to lower the yield of infectious Ad (Hoggan et al., 1966). Delayed onset of tissue culture cytopathology was also noted during an AAV-Ad infection (Hoggan et al., 1966). When anti-AAV serum was added to the mixed infection a reversal of the Ad inhibition was seen, implying AAV was the inhibitory agent (Hoggan et al., 1966). Characterization of AAV's inhibitory effect on Ad yield has been studied in the simian, human, bovine, porcine, canine and murine systems with inhibition detected in all cases where AAV could replicate. Through the use of an infectious center assay, it was determined that there was an 85% reduction in the number of cells yielding Ad (Casto et al., 1967). This inhibition was greatest when AAV was added 24 hours prior to Ad or at the same time as Ad but not if AAV was added 6-8 hrs after Ad. Ad plaques were also reduced in size by the presence of AAV. Characterization of the AAV inhibitory effects have shown vesicular stomatitis virus, vaccinia, poliovirus, and IBR (a bovine herpesvirus) were not inhibited but SV40 was inhibited (Casto

et al., 1967; Parks et al., 1968). Ad becomes refractive to AAV's inhibitory effects at the time Ad DNA biosynthesis begins (Parks et al., 1968). When AAV is added to a mixed infection at a very high particle-to-cell ratio, self inhibition of AAV is seen. This is believed to be due to inhibition of helper Ad, thus interfering with AAV replication. Recently Carter et al. (1979) have demonstrated the presence of defective interfering particles (DI) of AAV. The particles are noninfectious encapsidated AAV which band at a slightly lower density (1.32-1.35 g/cm^2) in CsCl and contain deleted snap/back genomes (Hauswirth and Berns, 1979). These particles can significantly lower the yield of AAV but not Ad. Ultraviolet treatment abolishes their interfering capacity. Replication of AAV DI particles requires both Ad helper virus and normally replicating AAV, which appears to provide AAV specific products such as capsid proteins for the defective particle (Cheung, 1979).

The inhibitory effects of AAV on Croup A Ad oncogenicity have been demonstrated in newborn Syrian Hamsters (Kirschstein et al., 1968; Mayor et al., 1973). AAV can also inhibit the transformation of hamster embryo cells by Ad (Casto and Goodheart, 1972), and the growth and metastases of herpes simplex virus transformed hamster embryo fibroblasts (Blacklow et al., 1978).

CHAPTER II

INHIBITION OF ADENOVIRUS TRANSFORMED CELL ONCOGENICITY BY AAV

Introduction

The adenoviruses (Ad) are double stranded DNA viruses that have a naked icosahedral capsid (Horne et al., 1959). The virion contains a $20\text{-}25 \times 10^6$ d double stranded DNA genome (Pina and Green, 1965). Ad DNA has two interesting physical features: 1) the two ends of the genome contain an inverted terminal repetition of approximately 100 nucleotides (Garon et al., 1972; Wolfson and Dressler, 1972; Steenbergh et al., 1977); 2) a 55K dalton protein is covalently linked to the 5' termini of both ends of the genome (Robinson et al., 1973; Rekosh et al., 1977).

The viral genome has a coding capacity for 30-40 polypeptides of which 10 structural proteins have been identified (Maizel et al., 1968a; Maizel et al., 1968b; Everitt et al., 1973). The genetic map of Ad (Ad2 and Ad5 are the serotypes best studied) is divided into two main classes of genes. "Early genes" are those genes expressed prior to the initiation of Ad DNA replication while "late genes" are those expressed following the onset of Ad DNA replication. The majority of the late genes are transcribed from one major promoter mapping at 16.4 units and result in 18 spliced mRNAs, most coding for structural proteins (Evans et al., 1977; Chow et al., 1977; Berget et al., 1977; Chow and Broker, 1978). Transcription of Ad early genes involves at

least 5 promoters (Wilson et al., 1979) and 18 mRNA species have been mapped (Berk and Sharp, 1977; Berk and Sharp, 1978; Galos et al., 1979; Philipson, 1979). These early transcripts map in 5 separate regions of the Ad genome with early region 1a and 1b (map position 1.4-4-5, 4.5-11) and 3 (76-86) on the r strand (rightward 3'→5') while early region 2A (75-62), 2B (29-11) and 4 (99-91) are on the l strand (leftward 3'→5').

Ad are very common viruses of humans that are associated with minor upper respiratory tract infections and conjunctivitis (Hilleman and Werner, 1954; Rowe et al., 1954). More interestingly, certain Ads are oncogenic in rodents and serve as a model system for studying the properties of transformed cells.

One current classification of human Ad is based upon their oncogenic potential in newborn Syrian hamsters. Group A (represented by serotypes 12, 18, and 31) are highly oncogenic, causing tumors within 2 months; Group B (represented by serotypes 3, 7, 11, and 16) are weakly oncogenic with tumors arising at 4-18 months; and Group C (serotypes 1, 2, and 5) are usually non-oncogenic (Trentin et al., 1962; Huebner et al., 1965). The viruses that fall into each of these groups tend to be related biochemically with the DNA of Group A having 48-49% G + C, Group B 49-52% G + C, and Group C 57-59% G + C as compared to mammalian DNA which is 42-44% G + C (Piña and Green, 1965). Members of the same group also share extensive sequence homology: Group A 85-90%, Group B 70-100% and Group C 85-95% compared to intergroup homology of 10-25% (Lacy and Green, 1965; Fuginaga et al., 1969; Green, 1970). Viruses from all three groups have the ability to transform cells in vitro (Freeman et al., 1967; Williams,

1973; Graham et al., 1977). Although Ad type 5 (Group C) is not itself oncogenic in newborn rodents, cultured cells can, under certain conditions, be transformed by this virus (Freeman et al., 1967). The transformed cells now have the ability to cause tumors in the appropriate host. H14b cells are an Ad5 transformed hamster embryo fibroblast (HEF) cell line produced by Williams (1973) (Goldman et al., 1974). These cells were isolated following infection of HEF by Ad5 ts 14 (late mutant) at the non-permissive temperature. Injection of H14b cells into 1-14 day old hamsters resulted in tumor formation by 6 weeks. The tumors that developed were small cell sarcomas with necrotic centers typical of tumors induced by the oncogenic Ad (Williams, 1973). The characterization of Ad5 DNA sequences in H14b cells using reassociation kinetics has demonstrated 5.5 copies of the left hand 40% of the viral genome per diploid amount of cell DNA. The analysis of Ad5 cytoplasmic mRNA's by reassociation kinetics maps these transcripts from 2-11 on the Ad5 genome (Flint et al., 1976; Flint and Weintraub, 1977).

Identification of the Ad proteins expressed in H14b cells was determined by the immunoreactivity of antibodies produced in the sera of hamsters bearing H14b tumors. The reactivity of these antibodies towards Ad proteins produced during lytic infection and in other Ad-transformed cell lines was compared in order to map the Ad transforming proteins. Early regions 1a and 1b seem to be all that is necessary for Ad transformation. The proteins that map in early region 1a are the 49K, 35-55K, 25K, and 11K while those mapping in 1b are 58K, 15K, and 14K (Levinson and Levine, 1977; Ross et al., 1980). The antigenic relationship between these proteins is not clear so it

is not known whether they are discrete polypeptides or related proteins sharing antigenic cross reactivities. The major Ad transformation protein found in virtually all transformed cells is the 58K Ad tumor antigen (T-antigen) and high titers of antibodies towards this protein are found in the sera of H14b tumor bearing hamsters. These findings are all in agreement with the mapping of the mRNAs expressed in these cells. Consistent with these data are studies relating the expression of the various Ad5 T-antigens and the amount of Ad5 DNA found in the transformed cells. Rat embryonic kidney cells transformed with the left hand 4.5% of the Ad genome (HpaI-E) express the early region 1a proteins. Cells transformed with the left 8% (HindIII-C) produce a 19K T-antigen in addition to the 1a products. Cells transformed with the left 15% (XhoI-C) which contains the entire early region 1a and 1b produce all of the Ad T-antigens found in cells transformed by total Ad DNA or virions including the 58K major T-antigen (Schrier et al., 1979). These studies clearly define the necessity for early region 1a and 1b in cell transformation by Ad. Detailed mapping of the mRNA's from early region 1a and 1b have detected four major spliced species (Berk and Sharp, 1978). Two promoters mapping at 1.5 and 4.5 map units are the proposed starting points for early regions 1a and 1b respectively (Wilson et al., 1979; for a review see Philipson, 1979). It is clear that the number of proteins identified in this region is far greater than the number of mRNA species identified, so further characterization of the mRNAs is necessary for a better understanding of the expression of Ad specific proteins found in transformed cells.

The ability of AAV to inhibit Ad oncogenicity in newborn Syrian hamsters was discovered during the testing of various Ad for oncogenicity. AAV was found to be a contaminant during some of these tests. When newborn hamsters were injected with human Ad12, 60% of the animals developed undifferentiated sarcomas by day 45. When AAV was mixed with Ad12 and injected into newborn hamsters 3% of Group 1 ($2.5x10^6$ infectious units [IU] of AAV) and 8% of Group 2 ($1.5x10^7$ IU AAV) developed tumors at 60 and 80 days respectively. The tumors were of the same histologic type as those given Ad12 alone (Kirschstein, et al., 1968). Mayor et al. (1973), using Ad31, were able to inhibit the incidence of tumors from 50% in the control group to 0% in those animals receiving AAV. AAV does not have an effect on SV40 oncogenicity in newborn hamsters (Mayor et al., 1973).

AAV also has the ability to increase the mean palpable tumor latency period of herpes simplex virus type 2 transformed hamster cell line 333-8-9. In these animals the incidence of lung metastases at day 70 was 50% in the group receiving cells only and 0% in AAV plus cells. Fluorescent antibody techniques indicated that AAV VP1 and VP3 were synthesized in these cells implying that they can serve as a partial helper for limited protein synthesis, even though no detectable HSV or adenovirus is present. This partial helper function depends on 333-8-9 cell passage number (Blacklow et al., 1978). Since AAV has the ability to inhibit certain types of oncogenicity, an epidemiological study looking for the presence of AAV antibodies in cancer patients was undertaken (Mayor et al., 1976). The results indicated that 75-85% of the normal adult population have antibodies to AAV2 or AAV3 while only 30% of matched cancer patients have these antibodies.

In patients with genital cancers (>90% show antibodies to herpes simplex virus) only 14% have antibodies to AAV2 or AAV3 compared to 85% in normal adults that have recurrent herpes infections. This study implies that there is a correlation between the lack of antibodies to AAV2 and AAV3 and the presence of cervical or prostatic carcinoma of presumed herpesvirus origin (Mayor et al., 1976).

We have been interested in determining the molecular basis of the ability of AAV to inhibit Ad oncogenicity. The H14b cell line is a typical Ad-transformed cell line in that it expresses various characterized Ad proteins, induces tumors when injected into newborn Golden Syrian hamsters, and behaves phenotypically like a transformed cell line. We have investigated the effect of AAV infection on the biological properties and on the expression of Ad-specific DNA, RNA, and proteins in these cells.

Materials and Methods

Cells

H14b cells (a gift of J. Williams; Williams, 1973) and DMBA-2R cells (a gift of R. Lausch) were maintained as monolayers in fortified Eagle's MEM containing increased concentrations of essential and nonessential amino acids, vitamins, 2 mM sodium pyruvate and 10% fetal bovine serum. DMBA-2R is a transformed hamster cell line developed from a 9,10-dimethylbenzanthracene induced tumor (Lausch and Rapp, 1971). HeLa cells in suspension were maintained in Eagle's spinner MEM (GIBCO) supplemented with 10% bovine serum.

Virus and Viral Infections

AAV were grown in HeLa cell suspension cultures with Ad type 2 as helper (Berns et al., 1975). AAV virions were purified from 48 hr infected cells by freeze-thaw disruption followed by sodium desoxycholate and trypsin treatment. The virions were banded three times in CsCl isopycnic gradients in an SW50.1 rotor at 4°C, 40,000 rpm for 20 hrs. After the third banding any remaining Ad's were destroyed by heating at 56°C for 15 min (Hoggan et al., 1966).

H14b and DMBA-2R cells were grown to 20-40% confluence, and washed with Gey's balanced salt solution (BSS) (Gey and Gey, 1936) prior to AAV adsorption ($2x10^3$ fluorescent focus units/cell) which was carried out at 37°C in fortified MEM supplemented with 2% bovine serum. After 2 hours excess virus was removed and the cells were fed with fortified MEM supplemented with 10% fetal bovine serum.

Transplantation of Cells

Twelve to fourteen day pregnant Golden Syrian Hamsters were purchased from Charles River Laboratories. Four to five day old baby

hamsters were injected subcutaneously (nape of the neck) with either 1-3x10^5 H14b cells or DMBA-2R cells in 0.1 ml Eagle's MEM. Experiments with DMBA-2R cells were done with one group of newborns and one group of adult hamsters. Starting two weeks after injection, animals were palpated for tumors at the injection site approximately every other day.

Clones in Methyl Cellulose

To measure anchorage independent growth, control or AAV-infected H14b cells were suspended in fortified Eagle's MEM with 10% fetal bovine serum and 1.17% methyl cellulose (1500 centipoise). Usually 1000 cells, suspended in 2.0 ml of methyl cellulose containing medium were placed in each well of Linbro FB6 multitest dishes in which 2.0 ml of medium with 0.5% Noble agar had been placed previously and allowed to gel. The plates were incubated for 14 days at 37°C in an atmosphere of 5% CO_2. The culture was placed under a photographic enlarger (9X magnification) and the projected clones counted.

Growth Curves

H14b cells growing in Corning plastic 75cm^2 tissue culture flasks were infected or mock infected with AAV. Twenty-four hours post-infection the cells were washed in Gey's BSS, trypsin and versene (disodium EDTA) treated for 1 min (0.1% trypsin; 0.04% EDTA in BSS), and suspended in medium at 10^5 cells/ml. The cell preparation was dispensed in 5.0 ml aliquots into a series of Corning plastic tissue culture flasks (25 cm^2 growing surface) such that the cells were seeded at 2x10^4 cells/ cm^2. At 24-hour intervals thereafter, the cells were suspended by trypsinization, diluted in medium and counted in a hemocytometer. Trypan blue (0.1%) exclusion was used to determine cell viability.

Detection of AAV DNA Replication

Confluent monolayers of H14b cells growing in Nunc 5 cm diameter plastic dishes were infected with 10 ffu AAV/cell and/or 10 pfu/cell Ad5. Eighteen hours later the cells were labeled with 100 μCi ^{32}Pi (New England Nuclear) in phosphate free MEM supplemented with 5% fetal bovine serum. Forty-eight hours later low molecular weight DNA was extracted from the cells by the procedure of Hirt (1967). After RNase A (20 µg/ml) treatment and three extractions with an equal volume of H_2O saturated phenol the DNA was ethanol precipitated twice and electrophoresed on a 1% agarose slab gel (45 cm x 17 cm x 3 mm) at 135V for 16 hours. The gels were autoradiographed at 4°C using Cronex 4 film (DuPont) and a Kodak X-omatic intensifier screen. Bands on the autoradiograph were compared to an ethidium bromide stained AAV DNA marker.

Indirect Immunofluorescence of T-Antigens

Immunofluorescent staining was carried out according to Pope and Rowe (1964) and Blacklow et al. (1967). Normal hamster serum (NHS) was obtained from normal adult hamsters and anti-T-antigen sera (anti-T serum, 14b) from hamsters bearing H14b induced tumors. The 75 hamster anti-T-antigen sera was a gift of Dr. Alex Van der Eb. All sera were obtained by cardiac puncture followed by centrifugation at 2000 rpm for 15 min to remove the cells. At 72 hours post-infection the cells growing on coverslips (11 mm x 22 mm) were acetone fixed at -70°C for 10 minutes and reacted with a 1:10 dilution of either NHS or anti-T-antigen serum for 30 minutes at 37°C. The cells were then washed three times in PBS at 37°C and reacted with fluorescein conjugated goat anti-hamster immunoglobulins (Cappel Laboratories,

Cochranville, PA). Excess conjugate was washed off with PBS at 37°C and fluorescence was viewed at 400X magnification using a Zeiss fluorescent microscope.

Southern Blot Analyses

Approximately $1x10^8$ H14b cells (control or 3 days post-AAV-infection) were incubated with pronase (1 mg/ml) in 25 ml of 0.5xSSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 0.5% SDS (sodium dodecyl sulfate) at 37°C overnight with constant shaking. The lysates were extracted twice with water saturated phenol and then ether. The extract was incubated with pancreatic RNase A (20 µg/ml) for 1-2 hours at 37°C. After incubation the DNA was re-extracted twice with phenol and ether and precipitated with ethanol. The DNA was dialysed extensively against 10 mM Tris, pH 7.4.

Ad DNA

Ad type 5 (a gift of H. Ginsberg) was grown in HeLa cell suspension cultures. Cells were infected with Ad 5 (10 pfu/cell) and centrifuged (1500 rpm, 15 minutes) 48 hrs post-infection. After freeze-thawing 3x the cells were disrupted with sodium desoxycholate and trypsin (0.6% DOC, 0.1% trypsin) and incubated at 37°C for 30 minutes. Cesium chloride was added to 4.5 ml of extract and adjusted to N_D = 1.3672 (index of refraction). The virus was banded in the SW50.1 rotor by spinning at 40,000 rpm for 20 hrs at 4°C. The virus band was collected by dripping fractions from the bottom of the tube and rebanded as described above. Purified Ad-5 was dialysed against 1x SSC overnight at 4°C. DNA was extracted from the purified virions by treatment with β-mercaptoethanol (0.35M) and papain (15 µg/ml) for 2.5

hrs at 37°C, followed by an overnight incubation with SDS (0.5%) and selfdigested (37°C, 1 hr) with pronase (1 mg/ml). The DNA was extracted with phenol and ether twice and dialysed exhaustively against 10 mM Tris·HCl, pH 7.4.

Restriction enzymes

All enzymes were purchased from Bethesda Research Laboratories (BRL) and digestions were carried out as described by the supplier.

Agarose gel electrophoresis

H14b DNA (10 μg) (control or 3 day AAV infected) was digested with restriction endonucleases and electrophoresed on 1% agarose (HGT) vertical slab gels (34x 17x 0.3 cm) for 16 hrs at 100V (35 mA). Electrophoresis buffer contained 40 mM Tris-HCl, pH 7.8, 1 mM EDTA and 5 mM potassium acetate. Control lanes contained 20 picamoles Ad5 DNA mixed with 10 μg calf thymus DNA as a carrier.

Blotting technique

Transfer of electrophoresed DNA to nitrocellulose required *in situ* denaturation of DNA by immersion in 1 M KOH for 30 minutes followed by neutralization by the addition of 1 M Tris·1M HCl for 45-60 minutes and titration to pH 6.9. The gel was washed for 45 min in 6xSSC, pH 7.0, and placed beneath a nitrocellulose filter (Schleicher and Schuell, BA85 0.45 μM pores) with blotting in the upward direction according to Southern (1975). After blotting overnight the majority of the DNA was transferred to the nitrocellulose. The filter was washed in 2x SSC and then baked for 2 hrs at 80°C in a vacuum oven.

Nick translation

Ad5 DNA was labeled with α ^{32}P-dCTP (specific activity > 300 Ci/mmole, New England Nuclear, Boston, Mass.) to high specific activity by the nick-translation procedure (Kelly et al., 1970; Rigby et al., 1977). The reaction mixtures contained 50 mM Tris·HCl, pH 7.0, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 50 µg bovine serum albumin, 0.1 mM each of dGTP, dATP, and TTP, and 0.3 nM of the α ^{32}P-dCTP, 2 ng of pancreatic DNase (Worthington), 5 units E. coli DNA polymerase I (Boeringer/Manheim) and 0.5 µg Ad5 DNA in a total volume of 100 µl. The reaction was incubated at 15°C for 45 min and stopped by the addition of EDTA to 20 mM and 100 µg calf thymus DNA. The reaction was heated to 68°C for 10 min and passed over a Sephadex G-50 column (1 cmx10 cm, Pharmacia Fine Chemicals) in 2xSSC and the excluded radioactive peak was collected. Specific activities of 1-3x10^8 cpm/µg were obtained.

Filter hybridization and autoradiography

Hybridization of the radioactive Ad5 probe to nitrocellulose was carried out by a modification of the procedure described by Denhardt (1966). Nitrocellulose filters were washed for 30 min at 37°C in 5xSSC containing 50% formamide, 4x Denhardt Solution (0.08% BSA, 0.08% ficoll 400 and 0.08% polyvinylpyrrolidone), 20 mM Tris·HCl, pH 7.4, and 0.5% SDS. The filters were then annealed for 72 hrs with the ^{32}P-probe (10 ng/ml) in an identical solution containing 50 µg/ml tRNA and alkali denatured probe. Following hybridization, the filters were washed twice in hybridization solution (minus probe) and once in 2xSSC for 30 min each at 37°C. The filters were dried and autoradiographed using Cronex x-ray film (DuPont) and a Kodak X-omatic intensifier screen at -70°C.

Northern Blot Analysis of Ad5 Specific mRNA in H14b Cells

RNA labeling

H14b cells at 20% confluence were infected or mock-infected with AAV ($2x10^3$ ffu/cell) and allowed to grow for 60 hours post-infection. The cells were then labeled with 5 μCi/ml ^{3}H-uridine (43 Ci/ mmole; Schwartz-Mann) for 12 hours. The cells were washed in ice cold PBS and removed from the 150 cm^2 Corning tissue culture flasks with a rubber policeman.

Total cellular RNA extraction

Cells ($3x10^8$) were lysed in 20 ml extraction buffer (0.15 M NaCl, 0.05 M sodium acetate, 0.005 M EDTA, 0.5% SDS, pH 5.1), and Dounce homogenized 2 strokes. Redistilled 85% phenol (20 ml) was quickly added, and the RNA lysate heated to 70°C for 30 minutes with frequent agitation. The mixture was cooled on ice and centrifuged to separate the phenol and aqueous phases. An equal volume of chloroform-phenol (1:1) and aqueous phase were shaken together at room temperature for 15 minutes and then centrifuged. The aqueous phase was precipitated with two volumes of ethanol and pelleted by spinning at 2400 rpm for 15 minutes. The RNA pellet was dissolved in 0.1 M sodium acetate, pH 5, and reprecipitated with two volumes of ethanol. The RNA pellet was stored under ethanol at -70°C. This procedure was adapted from Scherrer and Darnell (1962).

Separation of cytoplasmic and nuclear RNAs

AAV infected or control H14b cells ($1x10^8$) were suspended in 2 ml RSB (0.01 M NaCl, 0.01 M Tris, pH 7.4, 0.0015 M $MgCl_2$) and the nonionic detergent NP40 added to 1%. This mixture was placed on ice for 5 minutes and then spun at 3,000 rpm for 5 minutes. To the

supernatant fluid (cytoplasmic RNA) an equal volume of 2xSDS huffer (0.2 M NaCl, 0.01 M Tris, pH 7.4, 0.002 M EDTA, 1% SDS) was added and the mixture incubated with proteinase K (20 μg/ml) for 30 minutes at 37°C and then extracted three times with an equal volume of phenol-chloroform-isoamyl alcohol (24:24:1) followed by two extractions with chloroform-isoamyl alcohol (24:1). The cytoplasmic RNA was precipitated in 100 mM sodium acetate and two volumes of ethanol at -70°C.

Purified nuclei were extracted using the same procedure as whole cell RNA (Scherrer and Darnell, 1962).

Selection of polyadenylated RNA

Selection of polyadenylated RNA (mRNA) was achieved by passing the total RNA fraction through an oligo-dT column (type 3; Collaborative Research, Waltham, Mass.) in 0.5 M NaCl, 0.01 M Tris, pH 7.5, 0.5% SDS and 1 mM EDTA binding buffer. After three passes through the column, the column was washed extensively in binding buffer and then poly A containing RNA was eluted with 0.01 M Tris, pH 7.5, 0.05% SDS, 1 mM EDTA. This RNA was ethanol precipitated and stored at -70°C under ethanol (Aviv and Leder, 1972).

Methylmercury agarose gel electrophoresis

Agarose gels (1.5% HGT-Agarose, 19x15x0.3 cm) containing 10 mM CH_3 Hg OH and buffer (50 mM Boric acid, 5 mM $Na_2B_4O_2 \cdot 10H_2O$, 10 mM sodium sulfate, 1 mM EDTA) were electrophoresed at 150V, 60 mA for 4 hours (Bailey and Davidson, 1976). Each slot contained 10 μg of poly A containing mRNA. After electrophoresis the agarose gel was soaked in 0.5 M ammonium acetate and ethidium bromide (1 μg/ml) and photographed using Polaroid T57 film. All work was

performed under a chemical hood and all equipment in contact with methylmercury was soaked in 0.5 M ammonium acetate and 14 mM β-mercaptoethanol.

Preparation of diazobenzyloxymethyl paper (DBM-paper)

Aminobenzyloxymethyl (ABM)-paper was prepared as described by Alwine et al. (1977). A sheet of Whatman 540 paper (14x25 cm) was soaked in a 10 ml aqueous solution of 0.8 g of NBPC (3-nitrobenzyloxymethyl pyridinium chloride; BDH Biochemical, Carle Pace, NY) and 0.25 g sodium acetate. The paper was dried at 60°C and then baked at 130°C for 35 min. After two washes with water the paper was dried and then washed twice in benzene and air dried. ABM-paper was stored dessicated under a vacuum at 4°C in the dark. ABM-paper was activated to DBM-paper (the active form which binds denatured nucleic acids by their guanosine residues) just prior to use. The diazotization was achieved by soaking ABM-paper in 120 ml of a freshly prepared sodium nitrite solution (178 μg/ml, 1.2 M HCl) for 30 min at 4°C. This paper was washed five times with ice-cold water and twice with 25 mM potassium phosphate.

Northern transfer

The ammonium acetate soaked methylmercury gel was placed in a 50 mM NaOH bath to fragment the RNA within the gel. The gel was then washed in a 200 mM potassium phosphate bath followed by a 25 mM potassium phosphate bath. The gel was then blotted onto the DBM paper according to the procedure of Southern (1975) as previously described. The only difference was that 25 mM potassium phosphate was used as the blotting buffer. After 24 hrs of transfer the DBM-paper turned orange.

Hybridization

Prehybridization buffer (without probe) consisted of 50% formamide, 5xSSC, calf thymus DNA (1 mg/ml), 1% glycine and 5x Denhardt solution (minus BSA). Prehybridization was carried out for 5 hrs at 43°C. Hybridization of nick translated Ad5 probe was carried out as previously described with the following modifications: 1) 25 mM KPO_4, pH 6.5 was used, 2) five times Denhardt solution minus BSA was used, 3) calf thymus DNA (1 mg/ml) was substituted for the tRNA, 4) no SDS was present. Hybridization was carried out for 48 hrs at 43°C and the blots washed for 4 hrs in 50% formamide, 5xSSC at 37°C with three changes of wash buffer. This was followed by a 30 min 1xSSC wash. The blot was air dried and exposed to Cronex x-ray film (DuPont) as previously described.

Immunoprecipitation of T-Antigen

Cell extraction for immunoprecipitations were carried out using a procedure modified from Persson et al. (1978). AAV infected or control H14b cells were labeled with ^{35}S-methionine (10 μCi/ml) (∿1000 Ci/mmole, NEN) for 6-10 hours in methionine free MEM (GIBCO) supplemented with cold methionine (0.15 μg/ml) and 5% dialysed fetal bovine serum. The cells were washed in PBS, scraped from the surface and resuspended in buffer A (0.02 M potassium phosphate, pH 7.5, 0.1 M NaCl), 0.5% sodium desoxycholate, 1% Triton X-100, 0.1% SDS, and 1 mM phenylmethylsulfonyl fluoride (PMSF), and sonicated twice for 20 sec on ice. The extract was centrifuged at 12,000 g for 15 min at 4°C and the supernatant fluid was used as a source of T-antigen. The protein A antibody absorbent technique (Kessler, 1975; Ledinko, 1978) was used for isolating the T-antigen. Extracts (150 μl, standardized

to an equal number of cpm) were incubated overnight at 4°C with 10 µl normal hamster serum (NHS) or anti T-hamster serum. The extract was incubated with 150 µl of a 10% formalin fixed Staph A (New England Enzyme Center) for 15 min at room temperature followed by 45 min at 4°C. Antigen-antibody-Staph A complexes were spun at 2400 rpm for 15 min. The precipitate was washed 3x in buffer A, boiled for 5 min in elution buffer (2% SDS, 15% glycerol, 1% β- mercaptoethanol, 0.075 M Tris-sulfate, pH 8.4, and 0.001% bromophenol blue), and spun at 12,000 g for 5 minutes. The supernatant fluid was electrophoresed through a discontinuous SDS-polyacrylamide slab gel system (Laemmli, 1970) with a 5% stacking gel and 12.5% resolving gel (15x20 cmx1.5 mm) at 40 mA for 4.5 hours. After electrophoresis the gels were fixed in 7% acetic acid and 40% methanol and fluorographed using the salicylate treatment of Chamberlain (1979).

In control experiments H14b, 293 (Ad5 transformed Human Cells), and CHO cells were labeled for 9 hrs and HeLa cells either mock infected or infected with 30 pfu Ad 5/cell were labeled for 4 hrs at 9 hrs post infection. In these experiments the methionine free MEM was not supplemented with cold methionine.

Markers used on the SDS-polyacrylamide gels were either ^{14}C-labeled high molecular weight range package (Bethesda Research Labs, Inc., Bethesda, MD) which included Myosin (H-chain), 200,000 d; phosphorylase b, 92,500 d; bovine serum albumin, 68,000 d; ovalbumin, 43,000 d; α-chymotrypsinogen, 25,700 d; β-lactoglobulin, 18,400 d; cytochrome C, 12,300 d. Other markers used were ^{35}S-labeled extracts of poliovirus infected HeLa cells (a gift of Terry Van Dyke).

Results

Inhibition of H14b Tumors by AAV

Previous reports on the inhibition of adenovirus oncogenicity have involved the coinfection of AAV and Ad into neonatal Syrian hamsters (Kirschstein et al., 1968; Mayor et al., 1973). We have now been able to demonstrate inhibition of the oncogenicity of Ad-transformed hamster cells. When 1-3x10^5 H14b cells were injected into 4-5 day old hamsters, 75-100% (Table 1 Exp. 1-4, 7) developed tumors at the site of inoculation by 21-28 days. When 10^7 H14b cells growing in vitro were infected with AAV and subsequently injected into 4-5 day old hamsters only 17-46% (Table 1 Exp. 1-4, 7) of the animals developed tumors. This represented a significant inhibition of tumor formation (Table 1). The tumors that developed from AAV infected H14b cells had an increased latent period and in one experiment could not be palpated until day 45. These tumors tended to grow much more slowly and the tumor volumes were greatly reduced (1000x average). In many experiments the tumors just remained as small nodules. A histological comparison indicated that the two sets of tumors were histologically identical showing an undifferentiated morphology with central hemorrhagic necrosis.

The decreased oncogenicity of AAV infected H14b cells was dose dependent because when 10^5 AAV infected H14b cells were injected 14% of the hamsters developed tumors, but as the dose of cells was increased 10-fold the inhibitory effects were abrogated. Table 1 also shows the length of time the AAV-infected cells were grown in vitro before transplantation into hamsters. The time, noted in parentheses in the AAV column, did not significantly alter the inhibitory effect.

TABLE 1

INHIBITION OF AD-TRANSFORMED H14b CELL ONCOGENICITY BY INFECTION WITH AAV

Experiment	Cells	AAV	Tumors	%
1.	H14b	Yes(2hrs)	3/10	30
		No	18/21	86
2.	H14b	Yes(6hrs)	3/7	43
		No	7/9	78
3.	H14b	Yes(1 day)	1/4	25
		No	10/11	91
4.	H14b	Yes(3 day)	1/6	17
		No	3/4	75
5.	DMBA	Yes(1 day)	4/4	100
		No	4/4	100
6.	DMBA	Yes(1 day)	5/7	72
		No	4/5	80
7.	H14b 10^5	Yes(1 day)	1/6	17
	H14b $2x10^5$	Yes(1 day)	7/15	46
		No	17/17	100
	H14b 10^6	Yes(1 day)	8/8	100
		No	6/6	100

To test whether AAV could inhibit the oncogenicity of a non-adenovirus transformed cell line we used DMBA-2R cells. These dimethylbenzanthracene transformed hamster cells did not show a significantly decreased oncogenicity following AAV infection. Table 1, Exp. 5, represents adult hamsters infected with 10^4 DMBA-2R cells and all animals developed tumors. Table 1, Exp. 6, represents 4 day old hamsters injected with 200 DMBA-2R cells. Tumors developed in 80% of the controls and 72% of the hamsters receiving AAV infected cells.

In vitro Effects of AAV on H14b Cells.

Because AAV infection reduced the oncogenicity of H14b cells in animals, the effect of AAV infection on several of the biological properties of these cells in cell culture was also measured. A common characteristic of most transformed cells is anchorage independent growth. For normal cells to grow they must usually attach to a substratum such as plastic or glass; transformed cells on the other hand do not have this requirement and can grow in suspension cultures or while being suspended in a viscous environment like agar or methyl cellulose. This growth property is often correlated with the cells' oncogenicity (Freedman and Shin, 1974).

H14b cells demonstrated anchorage independent growth as measured by their ability to produce colonies when suspended in methyl cellulose. Two weeks after seeding H14b cells, large visible colonies approximately 1 mm in diameter were seen. Following an AAV infection, H14b cells also produced visible colonies by two weeks but the number of colonies was decreased (Table 2). These colonies appeared to arise at the same time and were the same size as the control cell colonies. Inhibition of colony formation varied from 38%-92% in five separate

TABLE 2

INHIBITION OF COLONY FORMATION IN
METHYL CELLULOSE CONTAINING MEDIUM

	Cells Plated	AAV*	No. Colonies	% Inhibition
Exp. 1.	10^3 H14b	No	254	
		Yes	120	53
Exp. 2.	10^4 H14b	No	1653	
		Yes	138	92
Exp. 3.	10^3 H14b	No	467	
		Yes	273	41
Exp. 4.	10^3 H14b	No	103	
		Yes	64	38
Exp. 5.	10^3 H14b	No	72	
		Yes	39	46

* $2x10^3$ ffu/cell

experiments (the reason for this variation is not known). Incubating the AAV-infected H14b cells for periods longer than two weeks did not increase their cloning efficiency.

In order to determine whether AAV infection killed H14b cells directly or had an effect on the growth of these cells, growth curves of H14b cells and AAV-infected H14b cells were compared (Figure 1). Both cell lines showed similar growth rates until three days post-infection at which time the infected cells started to grow at a slower rate. By five days the cells were confluent and stopped dividing. In all experiments the AAV-infected H14b cells appeared to grow to a lower saturation density and stopped dividing. If these cells were trypsin and EDTA treated to remove them from their growing surface and replated at a lower density the cells continued to divide until they reached a high density implying that the reason they stopped dividing was probably was not due to a general cytotoxic effect of AAV. Cell viability as determined by trypan blue exclusion was greater than 95% in all experiments.

Replication of AAV in H14b Cells

The decreased oncogenicity of H14b cells and the alterations in their biological properties after AAV infection might have been caused by AAV replication within the cells. Since H14b cells are an Ad transformed cell line expressing early Ad-specific genes, it was necessary to determine if AAV was replicating in these cells because of helper function provided by those Ad sequences being expressed. In order to detect AAV DNA replication, H14b cells were infected with AAV alone or AAV and Ad5. Hirt extracts of ^{32}P-labeled cultures were electrophoresed on 1% agarose gels and AAV DNA replication detected by

Figure 1.

Growth Curves of H14b and AAV Infected H14b Cells. Control H14b cells are represented by the triangles and AAV infected H14b cells are represented by the circles.

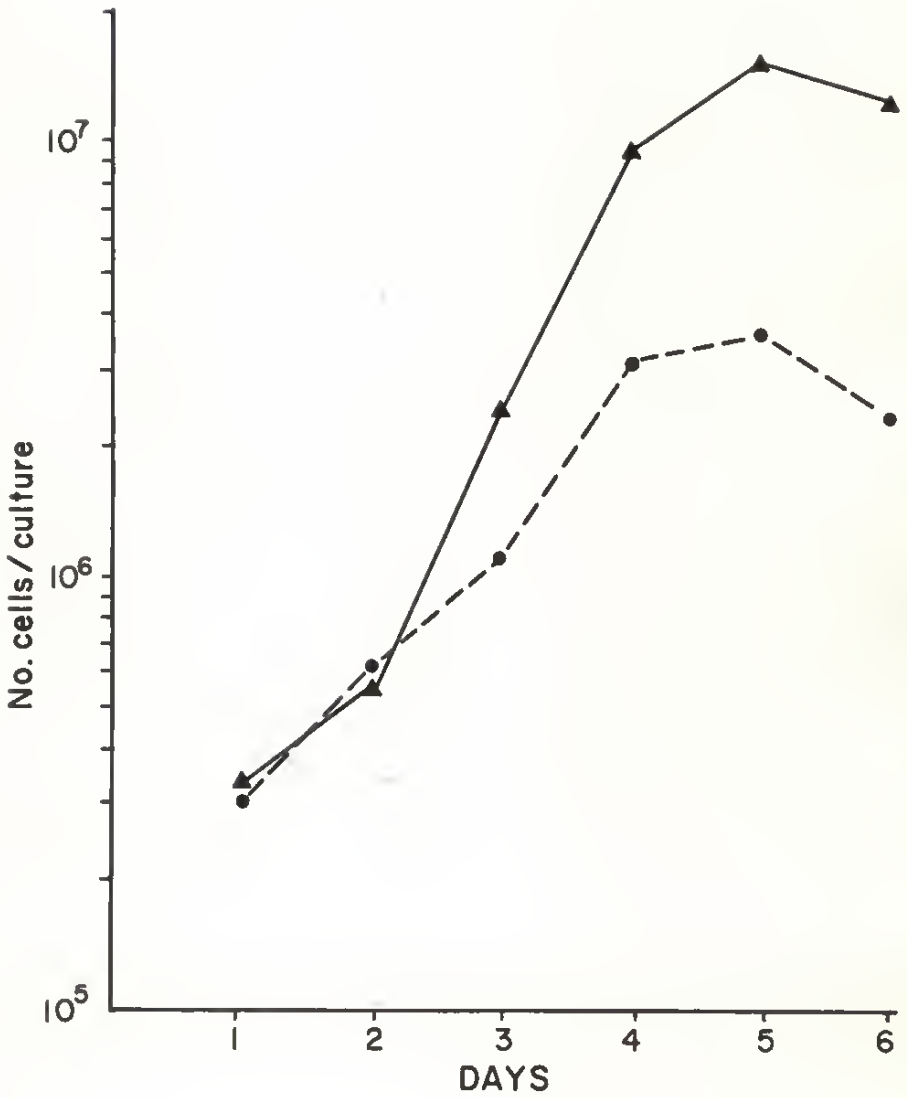

No. cells / culture
10^7
10^6
10^5
1
2
3
4
5
6
DAYS

Figure 2.

AAV DNA Replication in H14b Cells. Agarose gel electrophoresis of Hirt extracts from ^{32}P-labeled H14b cells infected with AAV alone, Ad and AAV, Ad alone, or control (C) cells. Replication of AAV DNA is represented by a band migrating to the position labeled AAV.


H14b CELLS
AAV
C
Ad
Ad
AAV
AAV

a band on an autoradiograph which comigrated with marker AAV. Figure 2 demonstrates that no AAV DNA replication could be detected in H14b cells infected with AAV alone or in the uninfected control, but when H14b cells were infected with both AAV and Ad5, AAV DNA replication was readily detectable.

Fluorescent Antibody Analyses of T-Antigen

The altered properties of Ad-transformed cells are presumably caused by viral-specific products that have been correlated with the presence of tumor specific antigens (T-antigen). To assess any changes in the T-antigens that may result from an AAV infection, H14b cells or AAV-infected H14b cells were grown on coverslips, fixed with cold acetone, and allowed to react with serum from normal hamsters (NHS) or hamsters bearing H14b tumors. The NHS serum is a control serum and should not contain antibodies directed against H14b cells. The serum from tumor bearing hamsters (anti-T antigen serum) contains antibodies against the Ad-specific gene products expressed by H14b cells, notably the T-antigens. Following the reaction of the cells with the various sera a fluorescein conjugated goat anti hamster immunoglobulin (IgG, IgM and IgA) was reacted with the cells and this indirect immunofluorescence assay was used to visualize the T-antigens. Figure 3 is a series of photographs taken to visualize the T-antigen immunofluorescence. Panel A shows H14b cells reacted with normal hamster serum and no immunofluorescence could be seen. Panel B shows the staining pattern of H14b cells that had been reacted with the anti T-antigen serum from tumor bearing hamsters. Nuclear fluorescence could be detected in these cells. The morphology of these cells is very rounded with a large nucleus and very little visible

Figure 3.

T-Antigen Immunofluorescent Staining Patterns of H14b Cells. Panel A represents control H14b stained with normal hamster serum. Panel B represents control H14b cells stained with anti-T-antigen hamster serum. Panel C represents AAV infected H14b cells stained with anti-T-antigen hamster serum.


C
B
A

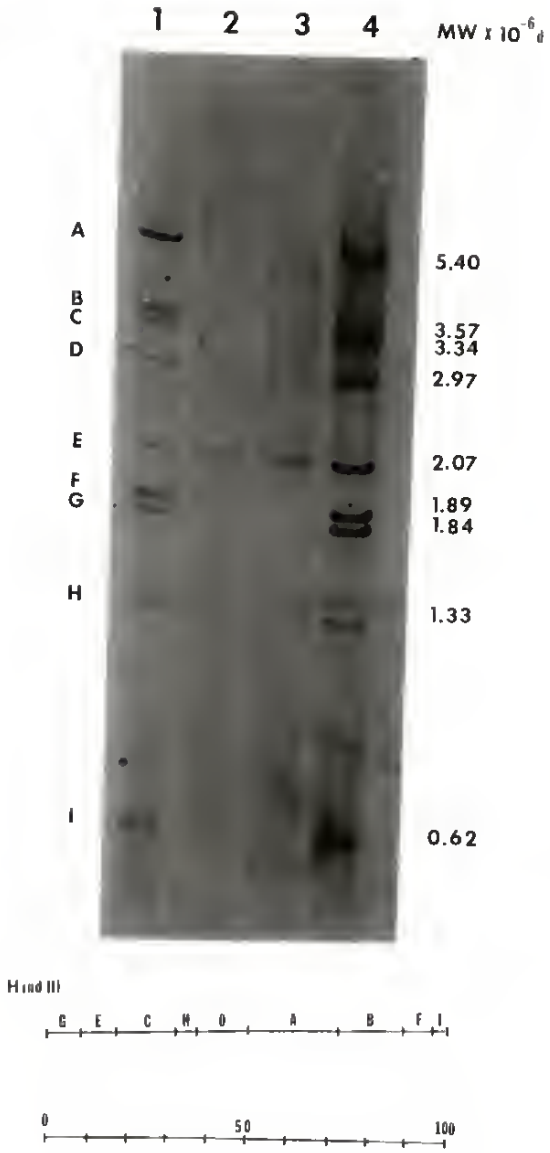
cytoplasm. Panel C shows AAV infected H14b cells reacted with the same anti-T antigen serum from tumor bearing hamsters. A significant reduction in the intensity of fluorescence could be seen. The amount of T-antigen available for reaction with the anti-serum may be reduced in AAV infected H14b cells.

Southern Blot Analyses of Ad DNA Sequences in H14b Cells

The reversion of the transformed phenotype in various virus transformed cells can often be associated with alterations or loss of the viral DNA sequences integrated within the cells (Groneberg et al., 1978; Basilico et al., 1979). The cellular DNA from AAV-infected and control H14b cells was analyzed using the blotting technique of Southern (1975) to determine if there were any alterations in the Ad DNA sequences. Figure 4 shows a Southern blot using the enzyme Hind III. In Ad5 DNA, Hind III makes eight cuts producing nine fragments ranging in size from $0.652-5.4 \times 10^6$ d. These nine fragments labeled A through I can be seen in Lane 1 and their map positions are shown at the bottom of the figure. Since H14b cells contain approximately 5.5 copies of the left hand 40%, restriction fragments C, E, C, H and the left hand 2.7% of D should all be present in these cells. If integration of Ad DNA occurs via the ends of the molecule, terminal fragments C and D may be attached to cellular DNA. When 10 μg of H14b cellular DNA (Lane 2) is digested with Hind III and analyzed by the Southern blot procedure using nick translated whole Ad5 DNA as probe, fragments E, C, and H can be detected. Three additional fragments, 1) smaller than A; 2) slightly larger than B; 3) larger than E, can be detected. A total of six Ad-specific cellular DNA restriction fragments can be detected. The analysis of 10 μg of AAV infected H14b

Figure 4.

Southern Blot Analysis of Ad DNA Sequences Contained in H14b Cells. Lanes 1 and 4 represent Hind III digests of Ad5 virion DNA. Lane 2 represents 10 μg of AAV infected H14b cell DNA. Lane 3 represents 10 μg of control H14b cell DNA. The ^{32}P-labeled probe is total Ad5 virion DNA.


1
2
3
4
MW x 10⁻⁶d
A
B
C
D
E
F
G
H
I
5.40
3.57
3.34
2.97
2.07
1.89
1.84
1.33
0.62
Hind III
G E C M O A B F I
0
50
100

cellular DNA using the same procedure (Lane 3) demonstrates that the identical six cellular DNA restriction fragments can be identified. This indicates that there is no alteration of Ad-specific DNA sequences occurring following AAV infection of H14b cells.

Northern Blot Analysis of Ad mRNA

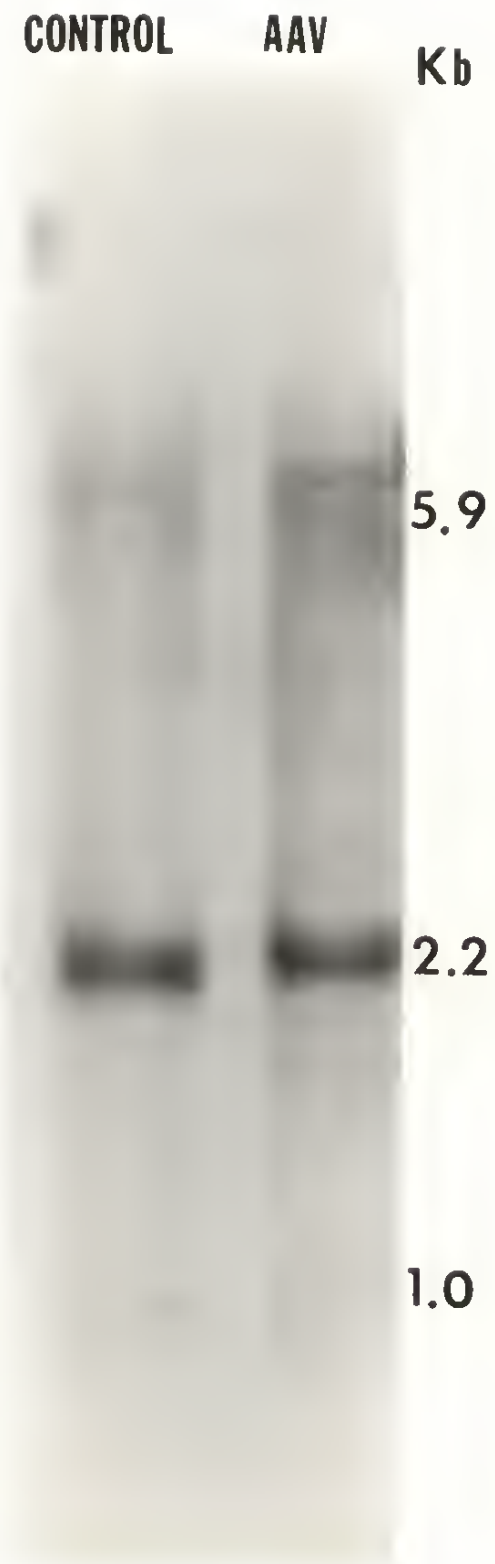
Whole cell RNA was extracted from control and AAV infected H14b cells and fractionated into poly A containing mRNA by passage through an oligo dT cellulose column. Messenger RNA (10 µg) was electrophoresed on 1.5% agarose-10 mM methylmercury hydroxide gels and the gel was blotted to DBM-paper. After hybridization of a ^{32}P-labeled nick-translated Ad5 restriction fragment Sal B (left hand 25%) probe, bands were visualized by autoradiography. A difference could be seen between control and AAV infected cells in the high molecular weight range of the gel (Figure 10). In the mRNA from AAV infected H14b cells an additional band could be detected at approximately 5.9 Kb. Three mRNA species were seen in this region of AAV infected H14b cells while only two species were detected in control cells. The major mRNA species detected were in the 2.2 Kb region where a doublet could be resolved. Other mRNA species of approximately 1.0 and 0.9 Kb could also be detected.

Immunoprecipitation of Adenovirus T-Antigens

Because fluorescent antibody analysis of Ad T-antigens demonstrated a noticeable decrease in the intensity of fluorescence following an AAV infection, we decided to directly analyze the presence of Ad T-antigens in H14b control and AAV infected cells. Immunoprecipitation studies were carried out on ^{35}S-methionine labeled H14b cell extracts and the immunoprecipitates were analysed on

Figure 5.

Northern Blot Analysis of H14b mRNA. Whole cell poly A RNA from control and AAV infected H14b cells were blotted onto to DBM-paper following methylmercury agarose gel electrophoresis. The blots were hybridized to ^{32}P-nick translated Ad5 Sal B restriction fragment (left hand 25%).


CONTROL
AAV
Kb
5.9
2.2
1.0

SDS-polyacrylamide gels. Figure 5 shows a fluorograph from an SDS-polyacrylamide gel. When normal hamster serum was reacted with the extract no specific bands could be detected. When sera from tumor bearing hamsters was reacted with the extracts, a doublet band in the 58K region of the fluorograph could be detected. In extracts from AAV infected H14b cells, the upper band of the extract was diminished in intensity implying less of the 58K tumor antigen was available for reacting with antibody. The same basic results were seen using two different sources of anti-tumor antigen sera, the 75 sera, from one hamster bearing an Ad5 induced tumors (a gift from Dr. Alex van der Eb) and the 14b sera, from hamsters bearing H14b cell induced tumors. Densitometer tracings of these gels allowed us to quantitate the amounts of Ad 58K tumor antigen precipitated. Figure 6 shows the tracings for the 14b sera (top) and 75 sera (bottom). These demonstate a significant inhibition in the levels of 58K Ad tumor antigen reacting with the antisera following an AAV infection. After repeating this experiment once and seeing the same inhibition of the upper band in the 58K doublet the source of ammonium persulfate was changed and the resolving 12.5% SDS-polyacrylamide gel no longer could resolve the doublet. Figure 7 shows a representative gel of an experiment repeated twice. It is clear that the 14b sera precipitated more 58K Ad tumor antigen from the control cells than from the AAV infected H14b cells. Quantitation of the densitometer tracings of this gel (Figure 8) demonstrates an 80% inhibition in the amount of T-antigen precipitated.

In order to ensure that we were indeed precipitating the Ad5 58K tumor antigen we immunoprecipitated ^{35}S-methionine labeled extracts from parallel cultures of HeLa cells, HeLa cells infected

with Ad5, H14b cells, 293 cells, and CHO (Chinese Hamster Ovary) cells. The left side of Figure 9 demonstrates that normal hamster serum (NHS) does not precipitate proteins from these cells while on the right side lanes 2, 3, and 4 which are Ad5 infected HeLa cells, H14b cells and 293 cells, respectively, all have a major 58K protein which precipitates and migrates identically. This shows that indeed the protein being inhibited following AAV infection of H14b cells co-migrates with authentic Ad5 tumor antigen.

Figure 6.

SDS-polyacrylamide Gel Electrophoresis. ^{35}S-methionine labeled immunoprecipitates of T-antigen were electrophoresed on 12.5% SDS-polyacrylamide gels. Lane C represents control H14b extracts and Lane A represents extracts from AAV infected (3 days) H14b cells. The control serum used was normal hamster serum (NHS) and the anti-T-antigen hamster serum were 75 and 14b. Markers on the gel were ^{35}S-labeled poliovirus proteins.

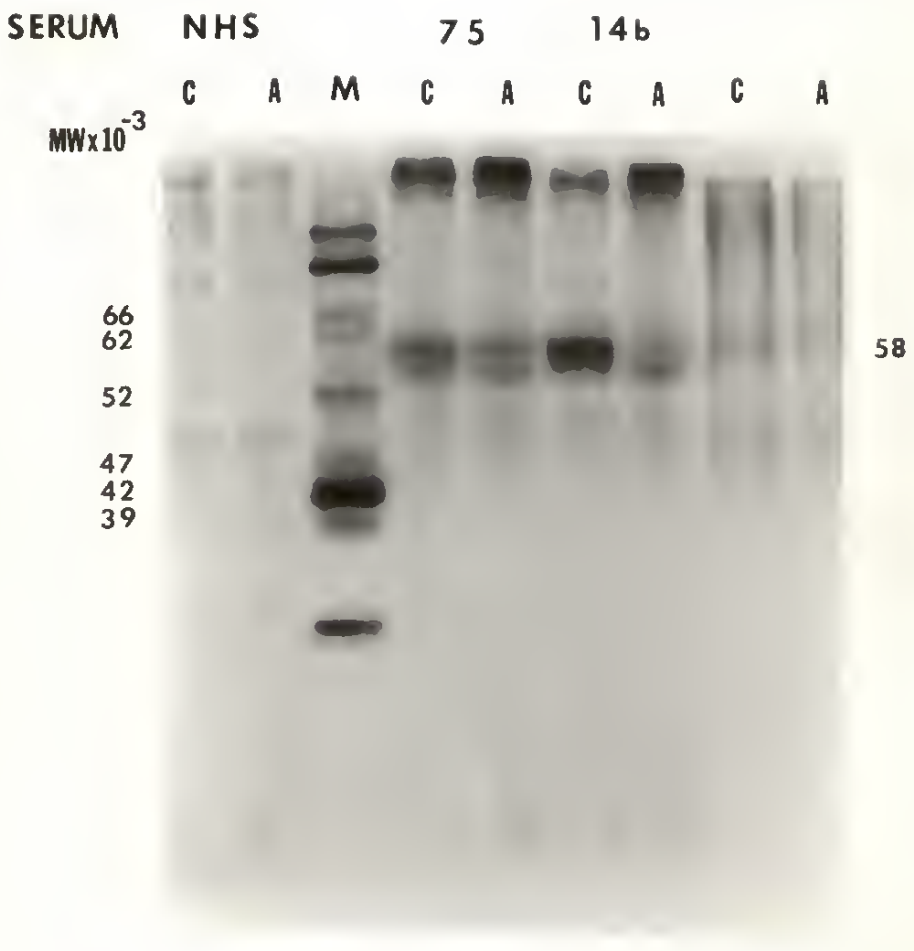

SERUM
NHS
75
14b
C
A
M
C
A
C
A
C
A
MWx10-3
66
62
52
47
42
39
58

Figure 7.

Densitometer Tracings of the 58K Doublet. Joyce-Loebl Densitometer tracings of the immunoprecipitates seen in Figure 5. The upper tracing is the 58K region from the 14b serum precipitate and the lower tracing is the 75 serum precipitate. The scan was right to left representing top to bottom.

58K DOUBLET

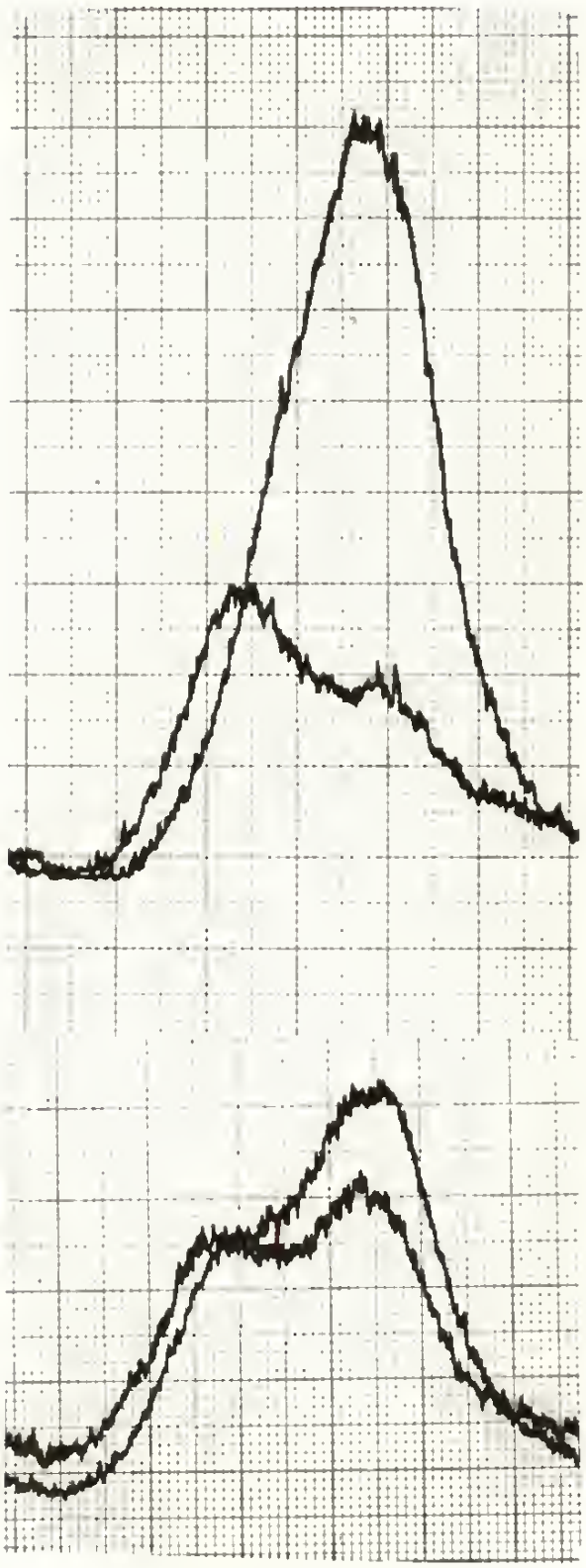


Figure 8.

SDS-Polyacrylamide Gel Electrophoresis. ^{35}S-methionine-labeled immunoprecipitates from control (C) and AAV infected (A) Hl4b cells were electrophoresed on 12.5% SDS-polyacrylamide gels. The precipitations were carried out with either normal hamster serum (NHS) or anti-T-antigen serum (14b). The center marker lane (M) is ^{35}S-labeled poliovirus proteins and the out marker lanes (M) are ^{14}C-labeled proteins (myosin, 200 K; phosphorylase b, 92 K; bovine serum albumin, 68 K; ovalbumin, 43 K; chymotrypsinogen, 25.7 K).

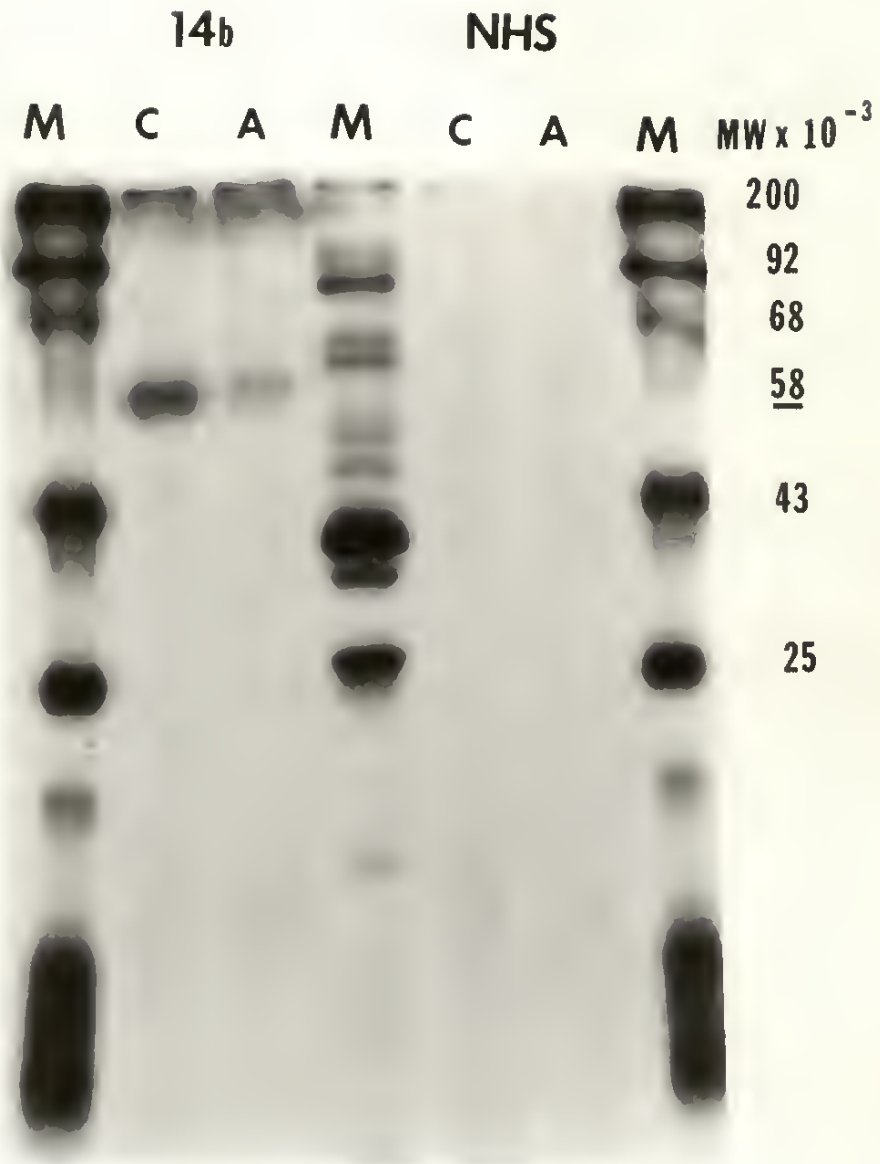

14b
NHS
M
C
A
M
C
A
M
MW x 10 -3
200
92
68
58
43
25

Figure 9.

Densitometer Tracings of Ad Tumor Antigen Immunoprecipitates. Joyce-Loebl Densitometer tracings of the immunoprecipitates seen in Figure 7. The tracings of the 14b C and A lanes are superimposed to quantitate the amounts of 58K tumor antigen precipitated. The right side of the tracing represents the top of the autoradiograph and the 58K tumor-antigen peaks are labeled 58K.

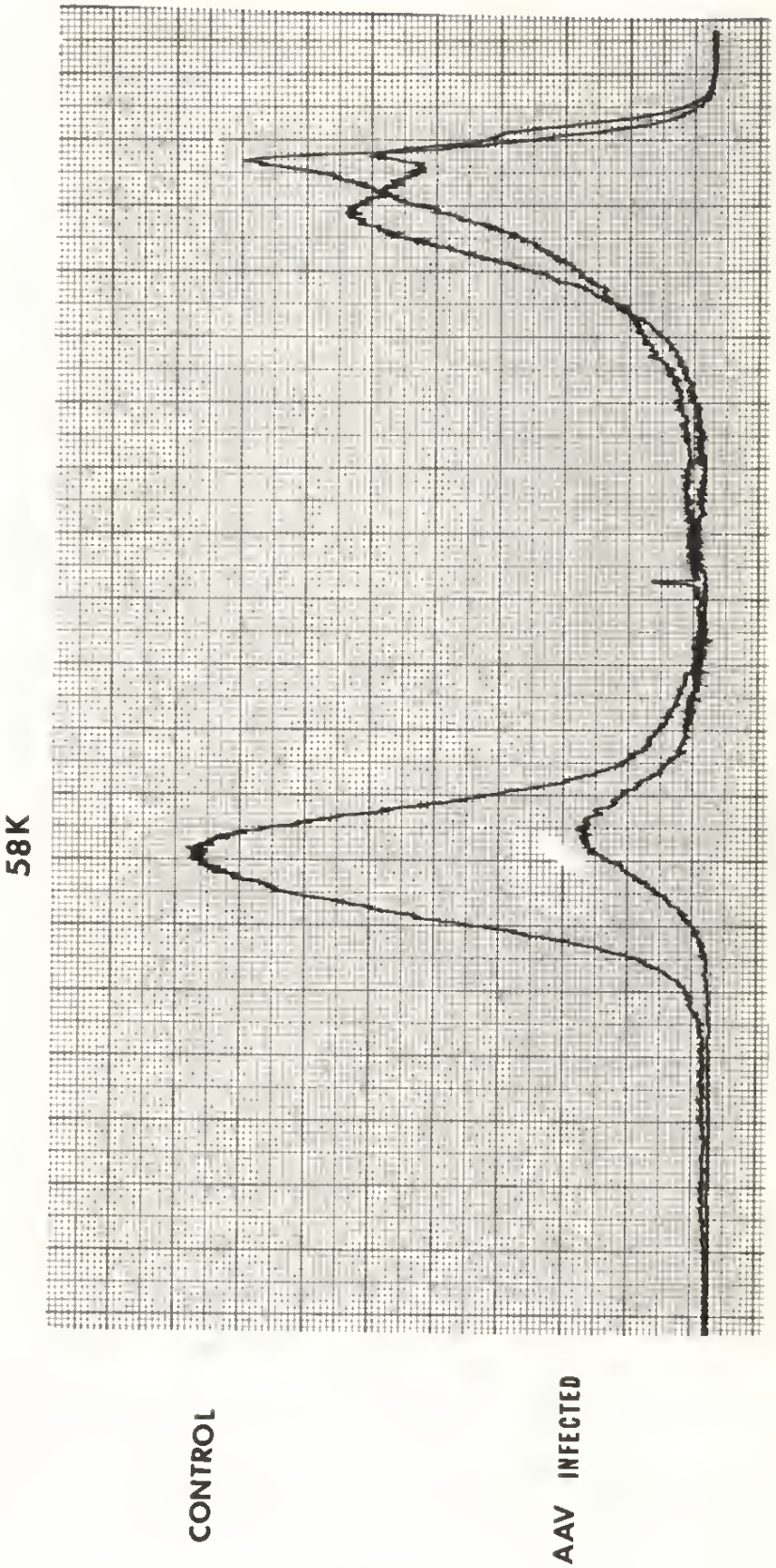
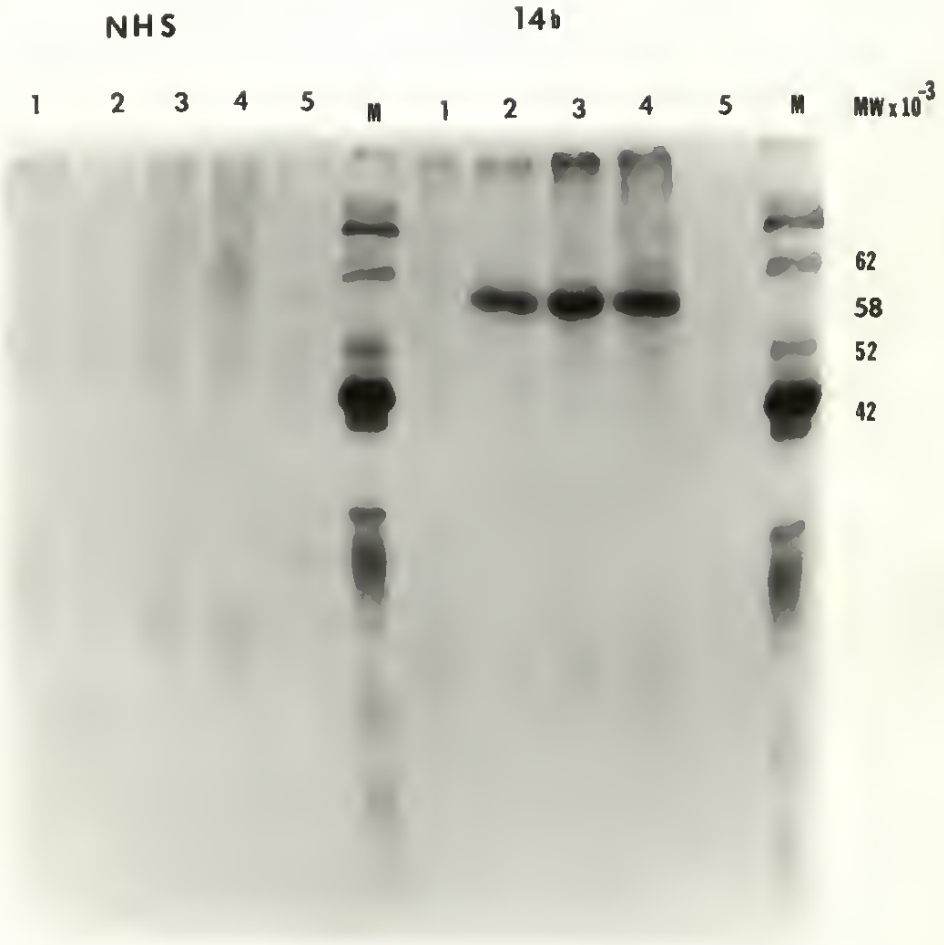

58K
CONTROL
AAV INFECTED

Figure 10.

Immunoprecipitation of Various Adenovirus T-Antigens. ^{35}S-methionine-labeled immunoprecipitates from various cell lines were analyzed by 12.5% SDS-polyacrylamide gel electrophoresis. The precipitations were carried out with either normal hamster serum (NHS) or anti-T-antigen serum (14b). The cell lines analyzed were HeLa cells (Lane 1), Ad5 infected HeLa cells (Lane 2), H14b cells (Lane 3), 293 cells (Lane 4), and Chinese Hamster Ovary (CHO) cells (Lane 5). Center lane markers (M) were ^{35}S-labeled poliovirus proteins and right lane markers (M) were ^{14}C-labeled proteins as in Figure 8.


NHS
14b
1
2
3
4
5
M
1
2
3
4
5
M
MW x 10^-3
62
58
52
42

Discussion

The elucidation of the molecular mechanism responsible for the ability of AAV to inhibit Ad oncogenicty involved the development of a model system to study these events. The Ad5 transformed hamster embryo cell line H14b served as a suitable example of an oncogenic cell line of which AAV could inhibit the *in vivo* and *in vitro* growth properties. Earlier studies on the ability of AAV to inhibit Ad oncogenicity were carried out by injecting AAV and Ad12 or Ad31 virus into newborn Syrian hamsters and determining the number of animals developing tumors (Kirschstein *et al.*, 1968; Mayer *et al.*, 1973). Studies of the mechanisms of inhibition of Ad oncogenicity could not be performed in this system due to the complex events occurring in the whole animal. Mechanistic studies in other Ad systems such as the ability of AAV to inhibit *in vitro* transformation by Ad were also not possible due to the length of time required for the establishment of the transformed phenotype as well as the very small percentage of cells in which the transformation event occurs. By utilizing the H14b cells we were able to develop a model system that overcame these difficulties.

When four to five day old newborn Golden Syrian hamsters were injected with uninfected H14b cells, 75-100% developed palpable tumors at the site of inoculation within two to three weeks. By two months these tumors accounted for a significant percentage of the hamsters' body mass. In animals receiving AAV infected H14b cells, only 17-46% developed tumors. The tumors that did arise often developed three to four days later than the control and grew more slowly and remained significantly smaller. The reduction of tumor incidence could be

overcome by injecting ten times the number of cells. Histological analysis of both tumors indicated that they were identical. These results indicate that AAV has the ability to inhibit the oncogenicity of the Ad5 transformed hamster cell line H14b. This inhibition may be specific for Ad transformed cells in that a line of dimethylbenzanthracene transformed cells (DMBA-2R) was not affected.

Studies to determine if AAV infection altered the in vitro properties of H14b cells in culture were performed. Following AAV infection colony formation in methyl cellulose was inhibited from 38-92% in five separate experiments. This large degree of difference in the inhibition can possibly be interpreted either as biological variation or a change in the cell line during passage (i.e. clonal expansion or cell death). Growth curves of H14b cells demonstrated that AAV infection induces a slower rate of growth three days post infection and grows to a lower saturation density. Cell viability remained greater than 95% indicating a general toxicity was not reponsible for these events. It is possible though that some cells are dying and not counted as adherent cells. Whether this inhibition is due to a reversal of the transformed phenotype or a metabolic alteration in the cells is not known. During one experiment the AAV inhibited H14b cells were subcultured and passaged in culture for three months. When AAV-infected cultures were subsequently infected with adenovirus and analyzed for AAV DNA rescue and replication, no AAV DNA could be detected implying that the AAV that had entered these cells originally was either degraded or lost in passage, and no stable relationship had been established (Ostrove, unpublished observation). AAV can replicate in H14b cells in the presence of added Ad but

helper function for AAV replication is not provided by the Ad genetic information expressed in the H14b cell line.

The in vitro effects of AAV on H14b cells demonstrated that this cell line is a good model system for analyzing the molecular events responsible for inhibiting effects of AAV. Three approaches were taken to further characterize the system and they involved the analysis of the Ad specific DNA, RNA and proteins found in these cells. Southern blot analysis of Ad DNA after digesting with Hind III and Xho demonstrated that the arrangement of these sequences does not change following AAV infection. These results demonstrated that AAV does not induce revertants of Ad transformed hamster cells. Studies on revertants of Ad12 transformed hamster cells have demonstrated changes and loss of Ad12 DNA sequences integrated into various cell lines; these revertant cell lines also show phenotypic changes in growth properties and loss of T-antigen staining (Groneberg et al., 1979; Eick et al., 1980). Analysis of T-antigen expression in H14b cells following AAV infection showed a reduction in the levels of T-antigen available for reaction with antibody. Indirect immunofluorescence demonstrated a decrease in intensity of the fluorescent staining pattern.

The reduction in the levels of T-antigen can possibly be explained by a number of different mechanisms. Synthesis of T-antigen can be inhibited at the level of transcription, post-transcriptional processing, translation or post-translational modification, all resulting in lower levels of T-antigen. Northern Blot analysis of whole cell mRNAs transcribed off the Ad genome in H14b cells demonstrates the presence of three size classes of transcripts. The 0.9

and 1 kilobase (Kb) transcripts found in both control and AAV infected cells are two transcripts identified in Ad5 infected HeLa cells and map in early region 1a. The protein products are not well characterized but they are expressed in transformed hamster cells (Berk and Sharp, 1978; Ross et al., 1980). A larger transcript of approximately 2.2 Kb is found in both cells. This may correspond to the 2.33 Kb or 2.38 Kb transcripts mapped in early region 1b and is believed to code for the 58K tumor antigen (Berk and Sharp, 1978; Ross et al., 1980). There does not seem to be any gross alteration in the levels of these transcripts, and in some Northern blots two bands in this region can be resolved implying that they may be the actual transcripts reported during a lytic infection. The only major difference seen following AAV infection is in the 5.9 Kb complex. Two bands can be detected in control cells and three bands can be resolved following AAV infection. No transcripts of this size have been reported in lytically infected HeLa cells, so these transcripts may represent either run off transcription products or products transcribed off a cellular promoter. They may not represent unspliced transcripts because when cytoplasmic enriched RNA is analyzed these bands are still present. Detailed analysis of the T-antigens, SDS-polyacrylamide gel electrophoresis of immunoprecipitates from ^{35}S-methionine labeled H14b cells were performed. These studies showed a reduction in the amounts of the 58K major Ad5 tumor antigen. In early studies a doublet 58K T-antigen was resolved and only the upper band was inhibited. A similar doublet has also been demonstrated by Wold and Green (1979) who showed that the partial protease digestion patterns of both bands were highly related or

identical but who offered no explanation for the doublet. Later studies utilizing a new lot of ammonium persulfate in the making of the polyacrylamide gel failed to resolve the doublet but still demonstrated the inhibition (80% as determined by densitometer tracings).

This system we have described appears to represent an ideal model system for studying the interactions between AAV and the transforming genes of Ad. The exact mechanism by which AAV causes the reduction in T-antigen levels can be speculated upon. A few general assumptions can be made based upon some observations of Carter (personal communication) in which he showed that either AAV DNA transfected into cells or defective interfering particles of AAV could inhibit Ad oncogenicity. These observations imply that AAV DNA mediates the inhibition and specifically the ends of the DNA because they are found in the DI particles (Laughlin et al., 1980; Hauswirth and Berns, 1979). If the ends of AAV DNA have the ability to interact with the transcription complex and alter or inhibit specific Ad transcription, this could account for the inhibitory effects of AAV on Ad oncogenicity. Another possible mechanism is that AAV DNA can interact directly or bind to the 58K protein and inhibit it from functioning. There are many examples of tumor antigen-DNA interactions like the sequence specific binding of SV40 T-antigen to sites on the DNA near the origin of SV40 replication (Tjian, 1978). This binding seems to play a regulatory role in the control of SV40 transcription (Alwine et al., 1977). If such a regulatory system exists in Ad, AAV may be inhibiting Ad oncogenicity by interacting with that system. There is also precedence for the interaction of AAV

with the Ad 58K T-antigen. A host range mutant of Ad5 (hr6) is unable to rescue AAV from latently infected Detroit 6 cells. This mutant fails to make the 58K T-antigen, implying that this protein may play a role in AAV rescue (Ostrove and Berns, 1980; see Chapter III).

CHAPTER III

ADENOVIRUS HELPER FUNCTION AND THE RESCUE OF AAV-2 FROM LATENTLY INFECTED DETROIT 6 CELLS

Introduction

The DNA containing viruses that replicate in the nucleus have the ability to integrate their DNA into the genome of cells infected under nonpermissive conditions (Kraiselburd et al., 1975; Ketner and Kelly, 1976; Botchan et al., 1976; Sutter et al., 1978; Marion et al., 1980). This integration sometimes results in the establishment of a transformed cell. The integration of a viral genome into a cell can also occur without any detectable changes. This latter example describes the relationship between AAV and various cell lines in which it can integrate.

AAV can enter into a latent infection in cell culture or in the whole animal. Certain lots of primary human embryonic kidney cells and African green monkey cells can release AAV following a coinfection with adenovirus (Hoggan et al., 1972). In this natural carrier state no infectious AAV virions can be detected suggesting a cryptic relationship between AAV and the cell genome may exist within these cells.

The in vitro establishment of cell lines latently infected with AAV has been accomplished in the laboratory by infection of Detroit 6 cells (a human cell line derived from sternal bone marrow, Berman et al., 1955) with 250 $TCID_{50}$/cell of AAV-2 in the absence of helper

adenovirus (Hoggan et al., 1973). After 39 passages in tissue culture these cells were cloned and 18 of 63 clones tested yielded infectious AAV upon coinfection with adenovirus. One of these clones was recloned and established as the B7374IIID5 latently infected Detroit 6 cell line used in these studies. It has been estimated by experiments using reassociation kinetics that there are five AAV genome equivalents per diploid amount of cell DNA (Berns et al., 1975). Following adenovirus infection of these cells, infectious AAV virions are produced. The DNA isolated from rescued AAV virions has been shown to be identical to AAV virion DNA by restriction enzyme (Cheung et al., 1980) and terminal nucleotide sequence analyses (Berns, Fife and Hauswirth, unpublished observation).

Latent infections of other cell lines such as KB or HeLa cells with AAV-1 can also be established (Handa et al., 1977). Using reassociation kinetics, it was estimated that each cell contained 4-6 genome equivalents of AAV-1. Network analysis of these cells indicated 80% of the AAV DNA sequences were associated with high molecular weight cellular DNA.

Recently, Cheung et al. (1980) have studied the molecular state of AAV-2 DNA sequences in B7374IIID5 Detroit 6 cells. In early passage cells multiple copies of the AAV genome were clustered at a single location and may exist as head to tail tandem repeats. In this clone the ends of the integrated AAV DNA are covalently linked to cellular sequences.

Both wild type adenovirus and herpesvirus can provide the necessary functions for the rescue and replication of AAV DNA and the expression of AAV RNA and proteins resulting in infectious AAV

production. To date the helper function(s) supplied by adenovirus have not been defined. All temperature-sensitive (ts) mutants of Ad, including the DNA minus mutants (H5ts149 and H5ts125) can help AAV DNA replicate at the non-permissive temperature (Straus et al., 1976; Mayor and Young, 1978). Recent studies, however, have suggested that AAV DNA replication may be reduced two to five-fold and infectious virion production 100-fold with H5ts125, an Ad5 mutant, with a ts 72K DNA binding protein (Myers et al., 1980). In addition to the Ad ts mutants, host range mutants (hr-mutants) which map in two early complementation groups have been described (Harrison et al., 1977). These mutants do not replicate in HeLa cells but do multiply in 293 cells. These are an Ad5 transformed human cell line that contains the left 12% and the right 9% of the Ad genome with transcripts mapping only from the left end (Graham et al., 1977; Aiello et al., 1979). Group I hr-mutants (hr 3) are DNA negative under non-permissive conditions and have the ability to abortively transform rat cells but no stable transformed cell lines have been established. Group I mutants map between 0-4.4 with the defect in early region 1a. At low multiplicity of infection (moi) Group I mutants do not express the genes coding for all other regions (i.e., E1b, E2, E3, etc.) but at high moi this defect can be partially overcome. Group II hr mutants (hr 6) can replicate their DNA as efficiently as wild type under non-permissive conditions but still cannot transform cells. These mutants map between 4.4-9 and are defective in early region 1b. HeLa cells infected with Group II hr-mutants fail to synthesize the 58K early protein which is believed to be an Ad T-antigen (Levinson and Levine, 1977; Ross et al., 1980). Late polypeptide synthesis in

Group II hr-mutants is also reduced. Both Group I and Group II hr mutants can be complemented by all known Ad ts mutants, implying that they map in individual genes. The properties of hr-mutants are summarized on Table 3 (Harrison et al., 1977; Graham et al., 1978; Lassam et al., 1978; Frost and Williams, 1978; Berk et al., 1979; Lassam et al., 1979).

We were interested in testing various mutants of adenovirus for their ability to help replicate AAV DNA during coinfection, and for their ability to rescue AAV from latently infected Detroit 6 cells. The use of ts mutants in the late genes would enable us to determine which, if any, late genes were involved in AAV DNA replication and rescue. In order to determine the role of the early gene product from region E2, the mutant H5ts125 with its ts 72K DNA binding protein was tested for its ability to help replicate and rescue AAV DNA. This mutant is of the DNA negative phenotype, so the 72K DBP is essential for Ad DNA replication. The use of the host range mutants enabled us to test the role of early region 1a and 1b in AAV DNA replication and rescue from latently infected Detroit 6 cells.

Materials and Methods

Cell Lines

The origin and properties of AAV-2 latently infected Detroit-6 cells (B7374IIID5) have previously been described (Berns et al., 1975) and were a gift from Dr. M.D. Hoggan (National Institutes of Health).

HeLa cells growing as monolayers were a gift from Dr. C.H.S. Young (Columbia University). Human 293 cells were a gift from Dr. F.L. Craham (McMaster University).

Detroit 6, 293 and HeLa cells were maintained as monolayers passaged biweekly in Eagle's minimal essential medium (MEM, CIBCO) supplmented with 10% heat inactivated bovine serum. HeLa cells were also grown in suspension cultures maintained in spinner-MEM (Microbiological Associates) supplemented with 7% bovine serum and passaged daily.

Viruses

Ad 5 temperature sensitive (ts) mutants were a gift from Dr. H. Cinsberg (Columbia University). This ts mutant was grown in HeLa cells at the permissive temperature of 32°C. The non-permissive or restrictive temperature for this virus is 39.5°C with the 39.5°/32°C yield (pfu) $< 10^{-5}$ (Cinsberg et al., 1974).

Ad 5 ts mutants used in this study

Host range mutants from Croup I (hr 3) and Croup II (hr 6) were a gift from Dr. F.L. Craham. These viruses were grown in 293 cells. The properties of these mutants are summarized on Table 3.

Infections

The various cell cultures were grown at 37°C, 5% CO_2 in 5.0 cm diameter plastic tissue culture dishes (19.6 cm^2; Nunc). When cells

reached confluence the cultures were washed with Gey's balanced salt solution and infection mixtures (0.2 ml in MEM plus 2% bovine serum) containing 6 pfu/cell of Ad or 20 fluorescent focus units of purified AAV-2 or both viruses were placed on the cells. Adsorptions were carried out for two hours at 37°C (wt) or 39.5°C (ts mutants) with gentle shaking of cultures every 15 min. Following adsorption the cells were fed with MEM containing 10% bovine serum.

Analyses of AAV DNA Replication

At 14 hours post-infection the cell cultures were labeled with carrier-free ^{32}P orthophosphate (100 μCi/dish; Amersham) in 5 ml of phosphate-free MEM supplemented with 2% bovine serum. At 48 hours post-infection the low molecular weight DNA was extracted from the cells by the procedure of Hirt (1967). The cell culture was lysed and preciptated overnight at 4°C in 2% SDS, 10 mM EDTA and 1 M NaCl, and then centrifuged at 17,000 xg for 30 minutes. The supernatant fluids were incubated with pancreatic ribonuclease A (20 μg/ml, Worthington Biochemicals) at 37°C for 2 hours, extracted three times sequentially with equal volumes of water saturated phenol and then ethyl ether, and precipitated with 2 1/2 volumes of 95% cold ethanol. Samples were electrophoresed in 1% agarose gels (17 cm x 45 cm x 3 mm) at 135V for 15 hours. The gels were exposed to DuPont Cronex 4 film with a Kodak X-omatic regular intensifying screen at 4°C. AAV DNA replication was detected by the presence of ^{32}P-labeled DNA comigrating with ethidium bromide stained AAV DNA marker. That the bands attributed to AAV DNA replication represented AAV DNA and not Ad DNA was checked by hybridization using the blotting technique of Southern (1975).

Results

Adenovirus Host Range Mutants Provide Helper Function for AAV DNA Replication in 293 Cells

The host range mutants from Group 1 (hr 3) and Group II (hr 6) are able to replicate in 293 cells (Ad 5 transformed human embryonic kidney cells) because 293 cells make a product which can complement the defect in the hr-mutants. When 293 cells were infected with AAV alone (Table 4) no helper function for AAV replication was expressed and AAV could not replicate. In order to determine if hr-mutants could help AAV replicate, 293 cells were infected with hr 3 and AAV or hr 6 and AAV. At 14 hrs post-infection the cultures were labeled with ^{32}P orthophosphate and at 48 hrs the low molecular weight DNA was extracted by the procedure of Hirt (1967) and analyzed by agarose gel electrophoresis and autoradiography. The data represented in Fig. 11 (Lane J.O. and F.G.) demonstrated that wild type Ad 5, stocks J.O. and F.G. (received from Frank Graham) can help AAV DNA replicate. It also showed that both hr-mutants could also provide the necessary helper function(s) for AAV DNA replication in 293 cells. It appeared that the level of helper function for AAV replication was similar for the wild type and mutant adenovirus if we assume that the Hirt extraction efficiencies were the same after infection with these viruses.

Rescue of AAV DNA from Latently Infected Detroit 6-Cells

Because the Ad hr-mutants could provide AAV helper function in cells permissive for their own replication we tested the ability of hr-mutants to replicate (rescue) AAV from Detroit-6 cells latently infected with AAV. These cells do not contain any Ad or Ad DNA (Berns et al., 1975) and should not be permissive for host range mutant

TABLE 3

HOST RANGE MUTANTS OF ADENOVIRUS 5

	Group I	Group II	Wild Type
Representative	hr 3	hr 6	Ad 5
Growth on 293 cells	+	+	+
Complementation by Ad ts Mutants	All Classes	All classes	
Ability to Transform rat cells	Abortive	None	Stable
DNA Synthesis	-	+ Similar to wt	+
Late polypeptide synthesis	blocked	reduced	normal
72K early protein	- reduced	+	+
T antigen (FA)	-	Abnormal	-
58K early protein	±	-	+
10.5K early protein	- very reduced	+	+
Map position of mutation	0-4.4	4.4-9	

Figure 11.

AAV DNA Replication in 293 Cells Infected with Host Range Mutants of Adenovirus. Autoradiograph of agarose gel electrophoresis of Hirt extracts from ^{32}P-labeled 293 cells infected with AAV and Wt Ad5 (two different stocks JO and FG), host-range mutant 6 (Hr6), and host-range mutant 3 (Hr3). Replication of AAV DNA is represented by a band migrating to the position labeled AAV.


AAV infected 293 CELLS
AAV
WT
jo
WT
fg
HR6
HR3
ADENOVIRUS 5

Figure 12.

Rescue of AAV DNA from Latently Infected Detroit 6 Cells. Autoradiographs of agarose gel electrophoresis of Hirt extracts from ^{32}P-labeled Detroit-6 cells infected with wild type (WT) Ad5 or host-range mutants Hr6 and Hr3. Rescue and replication of AAV DNA is detected by a band migrating to a position labeled AAV.

DETROIT 6 CELLS (AAV)

WT WT HR6 HR3

ADENOVIRUS 5

replication. Figure 12 demonstrates that wild type Ad 5 can efficiently rescue AAV from these cells and help replicate the AAV genome. Neither hr 3 or hr 6 had the ability to rescue and help replicate the AAV DNA in these Detroit-6 cells. Even prolonged exposure of the autoradiograph did not demonstrate any AAV DNA replication.

We have looked for the rescue and replication of AAV using the very sensitive blotting procedure of Southern (1975) (Materials and Methods, Chapter II). When Hirt extracted DNA from wt and hr 6 infected Detroit-6 cells was hybridized to nick-translated AAV probe (specific activity 3 x 10^8 cpm/ug), AAV size DNA can be detected only in the wild type Ad 5 infected cell, but not in cells infected with hr 6 (data not shown).

Host-Range Mutant 6 Can Provide Helper Function for AAV DNA Replication in HeLa Cells

The demonstration that hr 6 could not rescue and help replicate AAV from the latent Detroit-6 cells was quite interesting in light of the fact that hr 6 did not have a defect in its own DNA replication and could replicate its DNA as efficiently as wild type. In order to determine if there was some peculiarity about Detroit-6 cells infected with hr 6, we tested HeLa cells, a cell line known to be non-permissive for hr 6. After infection of HeLa cells with hr 6 or wild type Ad 5 the cells were ^{32}P labeled and the low molecular weight DNA analyzed as before. To our surprise hr 6 was able to help AAV DNA replication in HeLa cells (Fig. 13). The fact that hr 6 can provide all the helper functions for AAV DNA replication in HeLa cells but not in Detroit-6 cells latently infected with AAV implies that the

Figure 13.

Demonstration of Helper Function for AAV DNA Replication Provided by Host-Range Mutant 6. Autoradiograph of agarose gel electrophoresis of Hirt extracts from ^{32}P-labeled HeLa cells infected with AAV and Hr6 (Hr6), AAV and Wt Ad5 (WT), and AAV alone (AAV). Replication of AAV is detected by a band migrating to a position labeled AAV.

HeLa CELLS

HR6 WT AAV

defect in hr 6 may be involved in the rescue of AAV DNA from its integrated state. This defect in hr 6 is therefore not involved in AAV DNA replication.

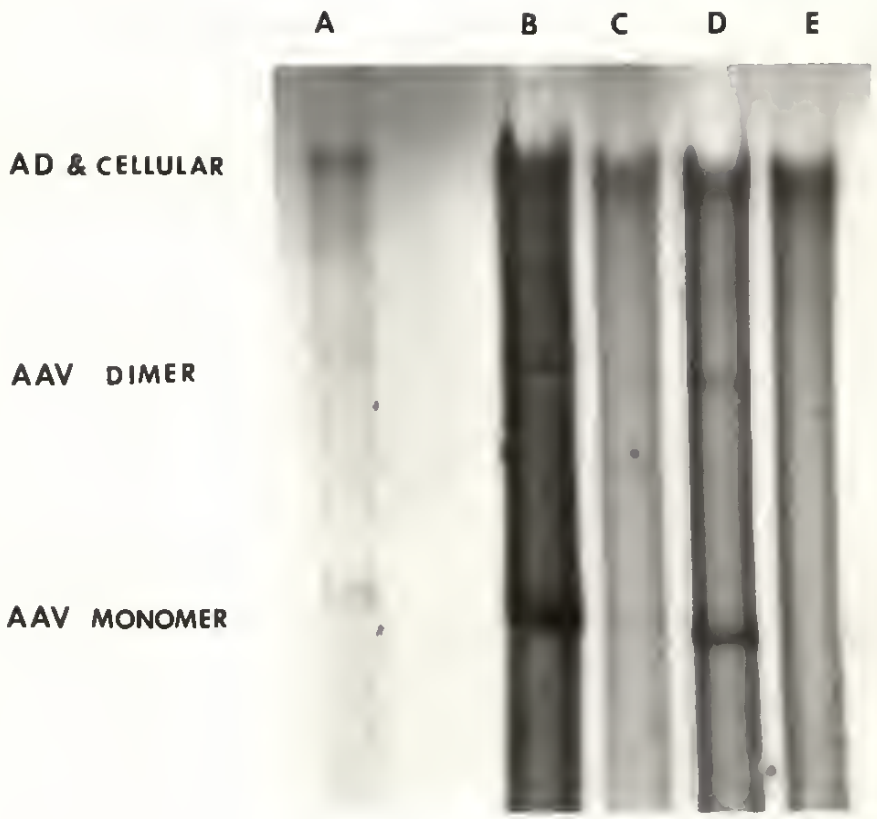
To determine whether hr 3 and hr 6 were behaving as they are reported to (Table 3) we tested hr 6 for its ability to replicate its own DNA and hr 3 for its DNA negative phenotype. ^{32}P-labeled Hirt supernates from hr 3 and hr 6 infected HeLa cells were hybridized to Eco RI digested Ad 5 DNA blotted on nitrocellulose filter according to the procedure of Southern (1975). Eco RI digested Ad 5 produces 3 fragments 76%, 16% and 7%. Autoradiographs of the blots demonstrate that wild type Ad 5 and hr 6 can replicate their DNA normally but hr 3 was unable to replicate its DNA (data not shown). These results are in agreement with published properties of the hr-mutants.

Replication of AAV DNA in Detroit-6 Cells Latently Infected with AAV

We have made the observation that hr 6 can help replicate AAV DNA in 293 cells (permissive) and HeLa cells (non-permissive), but not in Detroit-6 cells latently infected with AAV. Because this result implies that hr 6 is defective in rescuing AAV, it was necessary to determine if AAV DNA can be replicated by hr 6 when these latent cells are coinfected with purified AAV virions and hr 6. Figure 14 demonstrates that when latent Detroit-6 cells are coinfected with hr 6 and AAV virions (lane D), AAV DNA replication can occur normally, thus hr 6 can provide all helper functions necessary for AAV DNA replication, but is defective in its rescue of AAV from these latent cells (lane E).

Figure 14.

Demonstration of Rescue and Replication of AAV DNA by H5ts125, Host-Range Mutant 6, and Wild Type Adenovirus. Autoradiograph of agarose gel electrophoresis of Hirt extracts from ^{32}P-labeled cells. Lane (A) H5ts125 infected Detroit 6 cells (latently infected with AAV) grown at 40°C; (B) Ad5 hr6 and AAV coinfected HeLa cells; (C) Ad5 wt infected Detroit 6 cells (latently infected with AAV); (D) Ad5 hr6 and AAV coinfected Detroit 6 cells (latently infected with AAV); (E) Ad5 hr 6 infected Detroit 6 cells (latently infected with AAV).


A
B
C
D
E
AD & CELLULAR
AAV DIMER
AAV MONOMER

Replication and Rescue of AAV DNA by Early and Late Temperature Sensitive Mutants of Ad 5

We have tested various early and late ts mutants of Ad 5 for their ability to help replicate AAV DNA in 293 cells as well as their ability to rescue AAV from latently infected Detroit-6 cells. Below is a list of the ts mutants used and their defect:

a) H5ts125 (early) 72K DNA binding protein

b) H5ts135 (late) Assembly mutant

c) H5ts147 (late) Hexon transport mutant

d) H5ts116 (late) Hexon minus mutant

e) H5ts142 (late) Fiber minus mutant

Figure 15 demonstrates that the four late mutants tested all had the ability to help replicate AAV DNA during a coinfection in 293 cells at the non-permissive temperature of 39.5°C. These same mutants could all rescue AAV and replicate its DNA from Detroit-6 cells (Table 4). Thus, mutants in four late complementation groups all supplied the necessary helper functions for AAV DNA rescue and replication.

The early Ad 5 mutant, H5ts125, has a DNA negative phenotype at the non-permissive temperature. This mutant has a defective 72K DNA binding protein and cannot initiate or elongate its DNA during replication. When H5ts125 infects latently infected Detroit-6 cells under non-permissive conditions (39.5°C) AAV DNA could be rescued and replicated (Fig. 14, lane A), implying that the 72K DBP was not essential for rescue and replication of AAV DNA.

Figure 15.

Demonstration that Ad Helper Function for AAV DNA Replication is Provided by Various Temperature Sensitive Mutants of Adenovirus. 293 cells were infected with AAV and various temperature sensitive mutants of adenovirus and grown at the nonpermissive temperature of 39.5°C. Autoradiograph of an agarose gel electrophoresis of Hirt extracts from ^{32}P-labeled 293 cells. C (no Ad); H5ts147 (hexon transport mutant); H5ts142 (fiber minus mutant); H5ts135 (assembly mutant); H5ts116 (hexon minus mutant).


AAV infected 293 CELLS at 39.5 °C
AAV
C
ts147
ts142
ts135
ts116

TABLE 4

ABILITY OF ADENOVIRUS MUTANTS TO HELP OR INDUCE AAV DNA REPLICATION

	Cell Line			
			Detroit 6	
Infection	HeLa	293	1	2 (+exogenous AAV)
hr 3	-	+	-	-
hr 6	+	+	-	+
H5ts 125	N.T.*	N.T.	+	N.T.
H5ts 135	N.T.	+	+	N.T.
H5ts 147	N.T.	+	+	N.T.
H5ts 116	N.T.	+	+	N.T.
H5ts 142	N.T.	+	+	N.T.
Ad5 wt	+	+	+	+
AAV alone	-	-	-	-

* Not tested

Discussion

The results shown in Table 4 can be summarized as follows: 1) All late temperature sensitive Ad mutants tested supported the replication of AAV DNA in 293 cells. 2) All temperature sensitive Ad mutants tested rescued AAV DNA from latently infected Detroit 6 cells. 3) Without exogenous helper virus, AAV DNA was not replicated in 293 cells. 4) The Group 1 (hr 3) host range mutant supported AAV DNA replication in 293 cells, but not in HeLa or Detroit 6 cells. This mutant also did not rescue latent AAV from Detroit 6 cells. 5) The Group I1 (hr 6) host range mutants helped AAV DNA replication in coinfections of 293, HeLa and Detroit 6 cells, but did not cause detectable AAV DNA replication when added to latently infected Detroit 6 cells.

The various late Ad ts mutants were tested for their ability to help AAV DNA replication in 293 cells and rescue of AAV DNA from Detroit 6 cells because it seemed possible that certain late Ad functions might be required for AAV replication. This is probably due to the fact that Ad-transformed cells expressing only early Ad genes could not help AAV. Since these late mutants could both rescue AAV from Detroit 6 cells and replicate AAV DNA in Detroit 6 and 293 cells, it seems clear that the production of Ad fiber protein (H5ts142), hexon protein (H5ts116), the transport of hexon protein from the cytoplasm to the nucleus (H5ts147) or Ad virion assembly (H5ts135) are probably not necessary for any of the events involved in AAV DNA replication.

The Ad early mutant H5ts125 makes a defective 72K DNA binding protein under non-permissive conditions (39.5°C). At 39.5°C, H5ts125

does not replicate its DNA during an infection of permissive cells. When non-permissive cells are infected (i.e., rat cells) with H5ts125, these cells are transformed at a 3-8 fold higher frequency than wild type (Mayor and Ginsberg, 1977) and contain nearly the entire Ad genome integrated into their own genome. H5ts125 under non-permissive conditions (39.5°C) can rescue and replicate AAV DNA from Detroit 6 cells. These data imply that the 72K DBP is not essential for the rescue of AAV DNA, and probably not involved in AAV DNA replication. In view of the recent report that H5ts125 reduces AAV virion production by 100-fold and AAV DNA replication 2-5 fold (Myers et al., 1980) our procedures would not detect these slight differences in the amount of DNA replication. It is clear though, that AAV DNA can be rescued and replicated with H5ts125 under non-permissive conditions.

Most of the current data imply that Ad late genes are not necessary for AAV replication. This includes the replication of AAV in Vero cells microinjected with only early Ad mRNA (Richardson et al., 1980). In light of these observations it should be possible to find an Ad transformed cell line which has the ability to help AAV. In theory, 293 cells would be such a cell line because they are human cells expressing early region 1 genes. AAV alone does not replicate in 293 cells, but will replicate if coinfected with adenovirus. Other Ad transformed cell lines tested for their ability to replicate AAV DNA included H14b cells (Chapter II) and A18 rat cells which contain the entire Ad genome and express the majority of the Ad transcripts. None of these cells could help AAV DNA replication in the absence of Ad coinfection but helped normally during the Ad coinfection (A18, unpublished observation). It is clear from these data that it takes

more than those Ad early genes expressed in transformed cells to provide helper function for AAV. It may be speculated that certain cellular genes are induced during an Ad lytic infection which are required for AAV DNA replication, and these proteins are not present in Ad-transformed cells, but can be induced by coinfection with Ad. Another more likely possibility is that these proteins are just not expressed or expressed at very low levels in Ad transformed cells, and in order to replicate AAV DNA much higher levels of these Ad specific proteins are needed.

Both Group I and II host range mutants can provide AAV helper functions in 293 cells under permissive conditions for these viruses. Group I (hr 3) hr-mutants fail to support AAV DNA replication in a coinfection under non-permissive conditions such as in HeLa or Detroit 6 cells. Under the conditions tested, hr 3 does not replicate its own DNA (unpublished observation) and it is not clear which, if any, early genes are expressed. There is some uncertainty as to the details of the defect in Group I mutants. Berk et al. (1979) have suggested that only two pre-early mRNA species are transcribed in Ela. Lassam et al. (1979) have found evidence for the synthesis of some early proteins such as 58K and 72K. One probable conclusion is that a product not synthesized by Group I mutants is required for both Ad and AAV DNA replication, and this can be supplied by 293 cells. The failure of hr 6 (Group II) to induce AAV DNA replication in latently infected Detroit 6 cells is of particular significance. This result demonstrates that one or more additional functions must be supplied by the Ad helper specifically to rescue AAV from latently infected cells in addition to the Ad functions required for AAV DNA replication in a

normal coinfection. Thus other proteins besides those required for Ad DNA replication are necessary for the rescue of AAV. The Group II hr mutant can synthesize Ad DNA in HeLa cells at similar levels compared to wild type, but late polypeptide synthesis is reduced (Lassam et al., 1978, 1979). The defect in hr 6 seems to be in the expression of the early proteins mapping in early region 1b. Most notable is the loss of the 58K protein. This protein is believed to be the major Ad tumor antigen, and it is not produced in HeLa cells infected with Group II mutants. Group II infected cells have no T-antigen immunofluorescence staining (except one mutant, hr 51) and do not undergo transformation. Therefore, these data imply that the 58K Ad tumor antigen is necessary for the rescue of AAV from latently infected Detroit 6 cells, but is not necessary for AAV DNA replication.

Tumor antigens have been implicated in the rescue of SV40 and polyoma viruses from transformed cells (Botchan et al., 1979; Basilico et al., 1979). The viral DNA from cells transformed with temperature sensitive mutants in the large T antigen of SV40 and the gene A of polyoma cannot be rescued from these cells when grown under non-permissive conditions. This implies that these tumor antigens are required for the rescuability of integrated viral genomes in transformed cells. We have extended this observation to the adenovirus group demonstrating that the rescue of AAV from latently infected Detroit 6 cells requires the expression of the 58K Ad T-antigen (Ostrove and Berns, 1980).

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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